The Response to the Primary Systemic Treatment of Breast Cancer

Submitted for the degree of MD, University of Edinburgh, by Jeremy C. Keen, 1996

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ABSTRACT

The apparent heterogeniety of breast cancer in terms of natural history and response to treatment has limited the development of both new drugs and regimens. Primary systemic therapy has an advantage over standard post surgical adjuvant drug treatment in that the effects of treatment can be directly observed by monitoring the primary tumour response *in situ*. This concept underpins the studies described in this thesis.

The primary tumour response in terms of reduction in volume was monitored by serial breast ultrasound scans in 95 patients treated with the anti-oestrogen tamoxifen and in 15 patients treated with the cytotoxic agents adriamycin and cyclophosphamide. After three months of treatment 74% of patients treated with tamoxifen and 93% of those treated with chemotherapy had demonstrated a response.

Tumour response was monitored at a cellular level using immunohistochemical techniques to examine tumour biopsy material taken before and after treatment. Serial tumour fine needle aspirates were also taken during treatment from subgroups of patients undergoing either endocrine or cytotoxic therapy. These were analysed by flow cytometric methods that were initially validated in human cancer cell lines grown both in tissue culture systems and as xenografts. The results derived from patients receiving chemotherapy, whilst interesting, were limited by the small number of patients studied and an almost universally good response to treatment. However, larger numbers of patients and a broad spectrum of tumour responses produced more meaningful results in the tamoxifen-treated group. Several biological markers such as the expression of oestrogen receptors, *P*-glycoprotein, Ki-S1 and Bcl-2 were significantly related to the observed clinical response after multivariate analysis. Pre-treatment oestrogen receptor expression, measured by two different methods, correlated with the subsequent reduction in tumour volume after tamoxifen treatment with a high degree of significance. *P*-glycoprotein expression was significantly higher in post-treatment samples from tumours that had not responded to tamoxifen in comparison to responding tumours. This has potentially important implications for tumour sensitivity to second line treatment with certain cytotoxic agents. The expression of both Ki-S1, a nuclear antigen acting as a marker of proliferation, and Bcl-2, a marker of cellular resistance to cell death by apoptosis, were observed to significantly decrease in responding tumours. These effects occured independently and it is proposed that this may reflect two different pathways through which tamoxifen may exert its anti-tumour action, both by decreasing proliferation and/or increasing susceptibility to apoptosis.

CONFIRMATION OF ORIGINALITY

I confirm that the work contained within this Thesis is both original and my own except where the work of others is appropriately acknowledged.

Jeremy C. Keen, 1996

DEDICATION

To my wife Julie, daughter Laura Alexandra and my parents.

Do not be one of those who, rather than risk failure, never attempt anything.

Thomas Merton

INTRODUCTION

Chapter 1 : The Clinical Aspects of Breast Cancer - An Historical Review

1.1 The Epidemiology of Breast Cancer

Breast cancer continues to represent a major cause of mortality and morbidity in women in Scotland. Lung cancer has relatively recently become the greater cause of death but breast cancer continues to have the larger incidence, responsible for approximately 20% of all female cancer registrations. Given such a major problem, obviously much research has been engendered and although understanding of the disease has improved enormously there is yet to be significant improvements in overall survival rates. Indeed if incidence and mortality rates are examined there has been a persistent upward trend in both statistics during this century (Table 1.1).This trend is confirmed by epidemiological studies elsewhere in the world (Table 1.2) with Miller *et al.*(Miller, 1991) estimating an annual increasing incidence in the USA of 1.16 percent between 1940 and 1982. A faster rate of increase in incidence has been noted since 1982 and is thought to be mostly, if not entirely, due to the widespread introduction of mammographic screening. As a crude measure of the effectiveness of treatment it is interesting to plot the annual relative survival rates during the first five years after diagnosis (Fig 1.1), the relatively small differences between time periods are apparent.

Table 1.1 Age-Specific Death and Incidence Rates from Breast Carcinoma in Scotland (per 100,000 population).

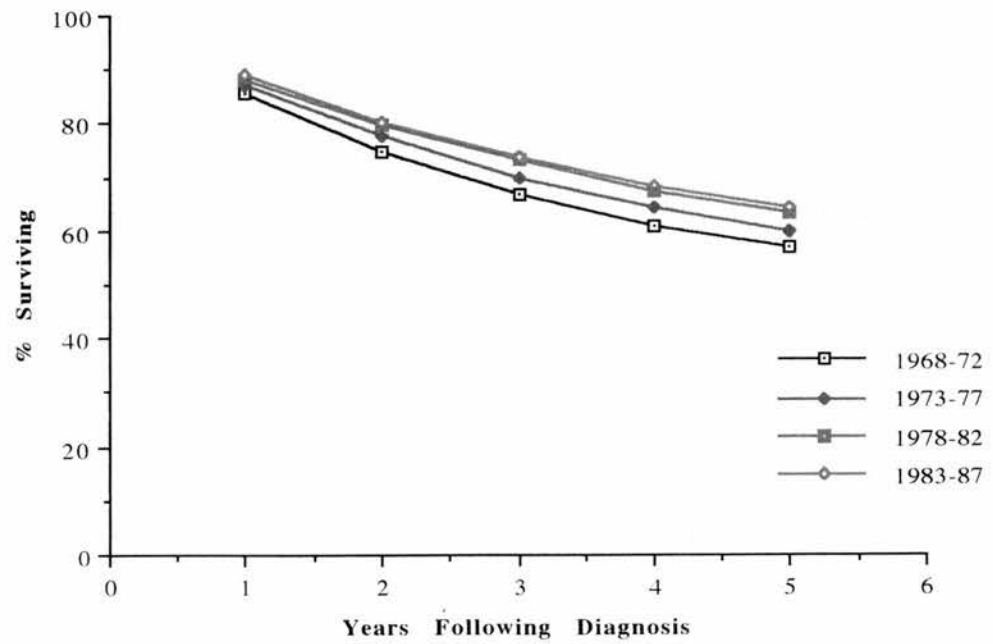
1911-15	**17.8**	1936-40	**27.6**	1961-65	**35.4 (57.7)**
1916-20	**19.0**	1941-45	**28.7**	1966-70	**38.3 (64.7)**
1921-25	**23.3**	1946-50	**29.1**	1971-75	**40.9 (71.6)**
1926-30	**23.3**	1951-55	**31.2**	1976-80	**42.5 (82.8)**
1931-35	**25.4**	1956-60	**33.8**	1981-85	**(92.9)**

figures in ( ) represent age-specific registration rates

Table 1.2 Age-Adjusted Breast Cancer Mortality Rates in Selected Countries

Country	1950	1955	1960	1965	1970	1975	1980	1983
England & Wales	23.2	23.7	23.8	24.4	26.4	27.8	28.2	28.4
Scotland	22.0	22.0	24.4	23.8	26.4	25.7	29.0	27.7
Northern Ireland	19.9	19.6	19.6	23.1	24.9	23.9	27.8	27.4
New Zealand	22.8	23.6	20.0	24.2	25.6	23.5	26.7	27.1
United States	21.3	22.0	21.7	22.2	22.5	21.9	22.0	22.1
Australia	19.4	20.4	18.7	18.7	19.9	19.4	19.2	20.4
France	12.7	14.4	16.0	16.9	17.0	18.8	18.3	19.2
Norway	17.3	18.6	17.0	18.3	17.0	17.4	17.5	16.7
Chile	7.7	7.6	9.6	9.8	12.1	11.4	12.1	12.7
Japan	4.0	4.0	3.8	3.9	4.4	5.0	5.5	5.9

Figure 1.1 Annual Relative Survival Rates in Scotland for Female Patients Diagnosed with Breast Cancer by Year of Diagnosis, 1968-87



Age is directly related to the incidence of breast cancer. The disease being relatively rare before the age of 25, climbing steadily through the premenopausal years, flattening at the menopause and then continuing to rise to peak in the over 80's. Incidence and survival rates can be studied in these age groups in an attempt to understand the underlying epidemiology and the relative effects of various preventative and therapeutic interventions (Table 1.3).

The figures shown in Table 1.3 are for Scotland only but the trends are similar in the rest of the United Kingdom. The number of new cases appears to have levelled out in all age groups other than in the over 75's, reflecting, in the most part, the increasing proportion of the general population in this older age group. Survival statistics show a gradual small improvement in all age groups but as already demonstrated this is most apparent over the first one to two years after diagnosis (see Fig 1.1).This may be explained in part by increasing public awareness and the introduction of breast screening leading to an increased proportion of patients presenting at an earlier stage in the disease. The overall impact of the widespread introduction of systemic cytotoxic and hormonal adjuvant therapy is difficult to assess. The improved survival of individual trial populations is clear but in relation to the breast cancer population as a whole the effects are relatively small.

The facts remain that there appears to be a continuing overall increasing incidence of breast cancer with, as yet, only minimal impact on survival from new therapeutic interventions. The rôle of clinical and laboratory-based research in breast cancer must therefore not only be directed towards the development of new therapies but also making the optimal use of existing drugs and techniques.

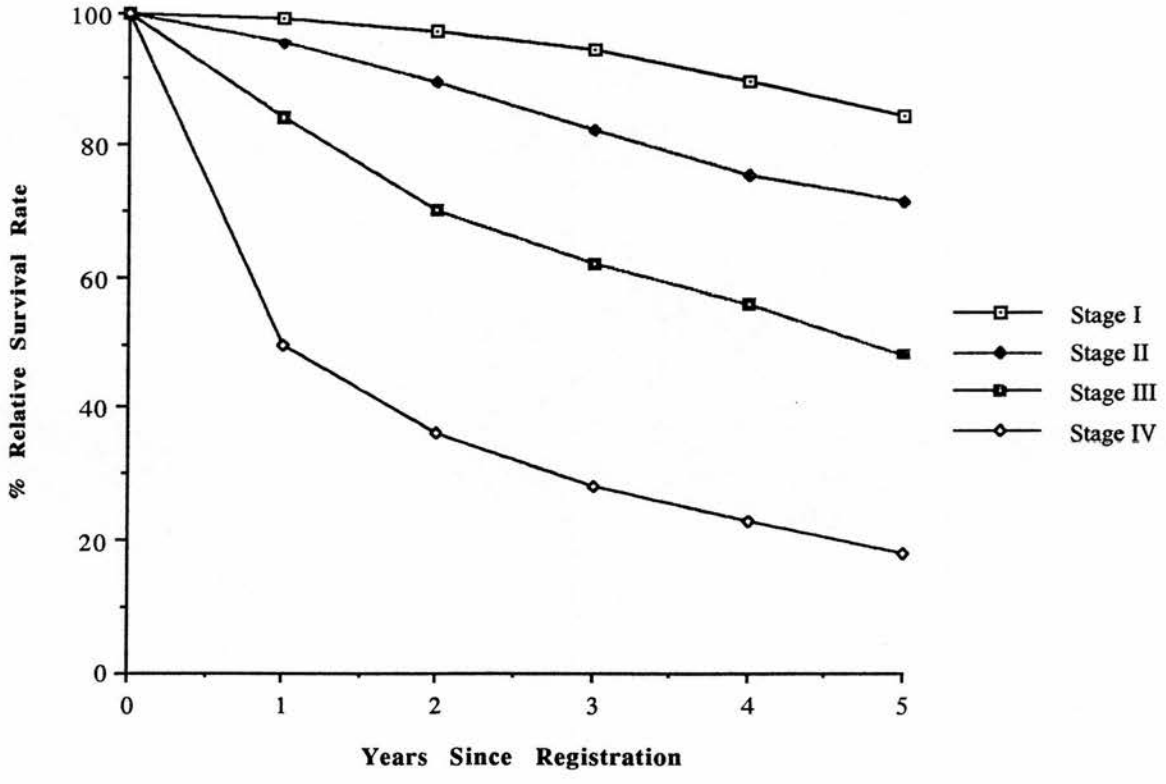
Table 1.3 Number of New Breast Cancer Registrations and Relative 5 Year Survival by Age Group and Year of Diagnosis (Scotland)

Age	Period of Diagnosis	Number of Registrations	Relative Survival Rate(%)	
			1 Year	5 Years
25-34	1968-72	243	87.3	55.0
	1973-77	266	91.4	57.4
	1978-82	269	93.8	64.6
	1983-87	253	94.5	63.1
45-54	1968-72	1997	87.9	57.8
	1973-77	2355	91.2	64.0
	1978-82	2295	91.5	64.2
	1983-87	2232	93.0	67.1
65-74	1968-72	2127	83.5	56.8
	1973-77	2676	84.6	57.8
	1978-82	2720	86.1	61.8
	1983-87	2544	87.6	63.1
75-84	1968-72	1202	75.2	52.7
	1973-77	1507	76.8	56.0
	1978-82	1687	79.3	59.8
	1983-87	1928	78.9	56.0

1.2 Clinical Staging and Prognosis

One of the most important factors determining survival from breast cancer has been shown in many studies to be the stage at which the disease first presents. Patients presenting with tumours confined to breast tissue with no evidence of local invasion or metastatic spread (Stage I) have an improved chance of survival compared to those with axillary lymph node involvement (Stage II) or local advanced tumours (Stage III), and much improved survival when compared to those with distant metastases at presentation (Stage IV, see Fig 1.2).

Fig 1.2 Relative Survival and Stage at Presentation (Patients from 1975-80)



(Thames Cancer Registry - Unpublished Data)

These obvious differences in survival between what is generally accepted to be early (Stage I) and late (Stage IV) disease form the basis for the rationale behind breast screening programmes and also the formulation of treatment protocols.

1.3 Current Concepts in the Treatment of Primary Breast Cancer

The treatment of primary breast cancer has until relatively recently been focused on the control of local disease within the breast by surgery and/or radiotherapy. In the 1930's Taylor was one of the first to suggest that ovarian oblation in addition to local therapy could improve survival in premenopausal patients with early disease (Taylor, 1934). However it was not until the mid 1970's with the publication of results of the first large scale trials of adjuvant chemotherapy that an understanding of the disease as potentially systemic, even at an apparently early stage, could be confirmed (Fisher, 1975; Bonadonna, 1976). Indeed it has been estimated that at diagnosis over 60% of patients will have either clinical or occult metastatic disease (Henderson, 1980). The importance of cytotoxic, hormonal and immune systemic therapy, in addition to local treatment, for improved clinical outcome has been reaffirmed by the recent report of the Early Breast Cancer Trialists' Collaborative Group (EBCTCG, 1992). The results of this report were based on the analysis of 133 trials carried out before 1985 involving 75000 patients treated by hormonal manipulation, principally with tamoxifen, and/or combined chemotherapy with CMF. Overall, in premenopausal women, adjuvant therapy with CMF brought about an annual reduction of risk of recurrence of 37% and tamoxifen a reduction of 11%. In postmenopausal patients the relative efficacys of the two forms of treatment were reversed with reductions of risk of 24% and 38% for CMF and tamoxifen (for 5 years) respectively. Clearly however there are significant groups of patients who gain very little or no benefit from such therapies and who are thus exposed to the immediate side effects of treatment and potential long term complications. Although avoidance of a net potential harm to patients is obviously the main factor in deciding against a particular treatment, preventing a possible misdirection of financial resources must also be considered.

With the acceptance that the underlying biology of an individual tumour may not only determine the potential for early metastatic spread but also the sensitivity to certain therapies, there have followed attempts to select those patients who will most benefit from a particular form of therapy. The two most accepted criteria are the axillary lymph node status (indicating a predisposition to metastatic spread) and the presence of steroid receptors (indicating less aggressive disease and a likelihood to respond to hormonal therapies). The EBCTCG report analyses results of therapy in these two patient sub groups and compared to the general study population notes a reduced risk of recurrence in node positive patients treated with chemotherapy and patients with oestrogen receptor positive tumours treated with tamoxifen. However the divide is not absolute and of particular importance is the improved outcome in a small but

significant group of node negative patients treated with adjuvant chemotherapy. This finding had been suggested previously and has spawned a vast amount of research aimed at identifying new prognostic indicators upon which to select patients for systemic therapy (See Chapter 5).

Developments in the approach to the local treatment of the breast have been directed towards less mutilating forms of local surgery. The similarity in treatment outcome between conservative local excision ("lumpectomy") followed by radiotherapy and total mastectomy is demonstrated by eight-year disease-free survival rates of 58% and 54%, respectively, for women with tumours up to four centimeters (Fisher, 1989) and 80% and 77%, respectively, for those with tumours up to two centimeters (Veronesi, 1986). These findings, in conjunction with other biological factors, discussed in the ensuing chapter, have encouraged the development of primary or "neoadjuvant" systemic therapy of primary cancers prior to surgery. In responsive tumours a decrease in size may allow breast conservation rather than mastectomy and encouraging results have been reported (Bonadonna, 1990).

Chapter 2 : Primary Systemic Threrapy of Breast Cancer

2.1 Historical Aspects

The clinical recognition of the importance of a combination of local and systemic treatment for breast cancer has stimulated developments in the understanding of both tumour growth and response to chemotherapeutic and hormonal agents. It is clear that there is a great diversity of short term responses and long term outcome to all agents used to date. This has been illustrated at a primary tumour level by the early trials of neoadjuvant chemotherapy employed principally to achieve response in locally advanced disease prior to surgery and/or radiotherapy (Hortobagyi, 1983; Swain, 1987; Jacquillat, 1988). Thomlinson attempted to document these varied responses by means of careful sequential measurements of primary tumours during treatment with chemotherapy, hormonal agents and radiotherapy (Thomlinson, 1987). With the possibility of monitoring the primary tumour *"in situ"* comes the potential to change therapeutic approaches if the desired response is not observed.This tailoring of therapy to individual tumours might be expected to have implications for all patients requiring sytemic therapy, not only those with locally advanced disease.The pilot study of primary sytemic therapy in Edinburgh (Forrest, 1986) included patients with operable but large tumours, given that the likelihood of the presence of micrometastatic disease is directly related to the size of the primary tumour (Ponten, 1990). In an attempt to spare patients the morbidity associated with chemotherapy all patients were initially treated with anti-oestrogens and their tumours monitored for response. Evidence of failure to respond or progression of disease led to a change in therapy to cytotoxic agents. The treatment protocol was revised so that those patients with oestrogen receptor poor tumours (<20fmol/mg cytosol protein) were initially treated by chemotherapy; this group having displayed a very poor response to anti-oestrogens. However within these two treatment groups there are still patients who will fail to respond or respond poorly to their assigned therapy.There is, therefore, a need to identify further prognostic markers, in addition to the oestrogen receptor, for therapeutic responsiveness and the human tumour model provides an ideal setting in which to pursue such studies.

There are other underlying biological reasons supporting the principal of pre-operative systemic therapy. Animal studies have shown that non-curative surgery accelerates tumour cell proliferation at metastatic sites (Tyzzer, 1973). This response can be blocked by treatment with cyclophosphamide or tamoxifen prior to surgery (Fisher, 1983). Commencing treatment as soon as possible may minimise the emergence of

mutations conferring drug resistance (Goldie, 1985). Avoidance of a burst of proliferative activity at surgery would also presumably reduce the risk of resistance mutation.

The early clinical studies of primary systemic therapy are now being reported (Table 2.1). The emphasis has necessarily, with short term follow-up, been on an end point allowing avoidance of mastectomy rather than a survival advantage. Indeed most studies have been prospective, with consecutive patient recruitment, comparing the response rates of different drug regimens. Interestingly similar studies have been undertaken in other cancers with the aims of allowing or facilitating local surgical and/or radiotherapeutic procedures. Examples include limb-salvage surgery in osteosarcoma (Goorin, 1985) and the preservation of the larynx (Hong, 1989) and bladder (Kaufman, 1989) affected by large invasive tumours.

Clearly the facilitation of local conservative treatment is an important outcome of primary drug therapy, perhaps particularly from a psychological viewpoint in breast cancer. The effect of this approach on recurrence and overall survival has yet to be confirmed. Although early survival data from the consecutive trials reported appear promising, there are, as yet, very few reported randomised comparitive trials of conventional local treatment and subsequent adjuvant therapy against primary systemic therapy followed by local treatment. Hopefully the multicentre NSABP (Protocol B-18) trial commenced in the U.S.A. and Canada in 1988 will include sufficient patient numbers to allow the questions of survival to be answered. A single centre French trial reported in 1991, included 272 patients with a median follow-up of 34 months and found an increased incidence of local recurrences in the primary therapy arm but an improved overall survival (Mauriac, 1991). However there were differences in the local therapeutic procedures employed with all in the conventional arm receiving a mastectomy while those in the primary therapy arm received a either a mastectomy, a wide excision followed by radiotherapy or radiotherapy alone. Interestingly there also appeared to be greater clinical responses to chemotherapy in oestrogen receptor negative tumours, which may fuel an argument for selected primary endocrine or chemotherapy. The current Edinburgh trial, upon which part of this thesis is based, was designed to help answer questions of disease-free and overall survival with standardised local therapy and randomisation of selected primary therapy versus conventional adjuvant therapy.

2.2 The Scottish Trial of Selective Primary Sytemic Therapy for Large Primary Breast Tumours in Women Under the Age of 70 Years

The early survival data resulting from the use of selective primary systemic therapy in a consecutive series of 88 patients presenting to the Edinburgh Breast Unit with large but operable tumours (>4cm), proved worthy of further investigation (Anderson, 1991). A pilot randomised controlled trial of primary systemic therapy versus conventional treatment has been conducted in the Unit since January 1990. The trial aims to compare the outcome of primary treatment, selected on the basis of tumour oestrogen receptor content and followed by locoregional therapy with standard postoperative unselected adjuvant systemic therapy.The measures of outcome are local recurrence rate, distant disease free interval and overall survival. Unlike other studies of primary systemic therapy previously quoted, potential downgrading of surgery required is not a factor in this study and all patients are selected for a particular procedure before commencing therapy. The design of the trial is outlined below (Fig 2.1) and the main inclusion/exclusion criteria include the following:

INCLUSION

i) Patients under the age of 70 years.

ii) Tumours greater than 3cm in clinical diameter.

iii) Stage T_2 or T_3 disease only.

iv) No evidence of distant metastases with standard staging investigations.

EXCLUSION

i) Patients with bilateral primary breast cancer.

ii) Patients with in-situ carcinoma only.

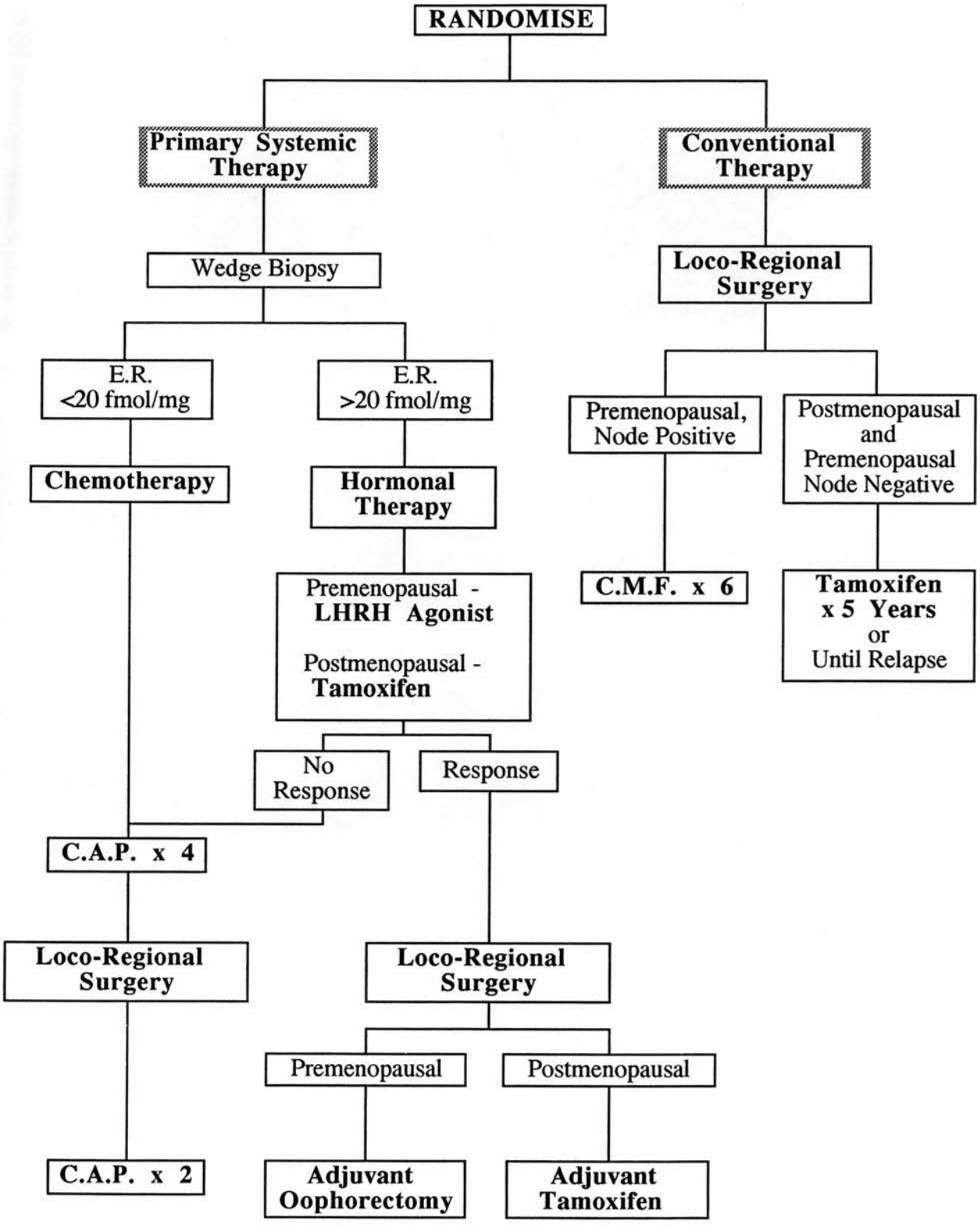
iii) Previous malignant disease (other than basal or squamous cell carcinoma of skin or carcinoma-in-situ of the cervix).

iv) Tumours that cannot be monitored adequately by mammography or ultrasound.

v) Patients with any medical condition that precludes adequate surgery, adjuvant therapy or follow-up (eg. pregnancy, heart disease, mental illness).

vi) Premenopausal patients with a wish for a future pregnancy.

Fig 2.1 Trial Design



Primary Therapy Arm

Patients randomised to the primary sytemic therapy arm of the trial undergo wedge biopsy of the tumour under local anaesthetic. Approximately 0.6cm^3 of tumour tissue is removed. A portion of tissue is fixed for histology and the remainder stored in liquid nitrogen for oestrogen receptor assay and further immunohistochemical and biochemical studies.
Primary therapy commences after histological confirmation of the diagnosis and the result of the oestrogen receptor analysis is known. Treatment is based on oestrogen receptor and menopausal status, lasts for three months, and is as follows:

a) Premenopausal patients with tumours of ER level >20 fmol/mg cytosol protein commence the LHRH agonist, Goserelin, by subcutaneous implant every 28 days. If a significant response is achieved a bilateral oophorectomy is carried out at the time of definitive breast surgery or shortly afterwards.

b) Postmenopausal patients with tumours of ER level >20 fmol/mg cytosol protein commence tamoxifen, 20 mg per day. In those achieving a response, tamoxifen is cotinued for five years after definitive surgery or until relapse.

c) All patients with tumours of ER level <20 fmol/mg cytosol protein commence CAP combination chemotherapy comprising:

Cyclophosphamide 1gm/m^2 I.V. bolus.
Adriamycin 50mg/m^2 I.V. bolus.
Prednisolone 40mg daily for 5 days orally.

Four cycles of therapy are given three-weekly prior to definitive surgery and if a response is demonstrated two further cycles are given after surgery.

d) Patients receiving endocrine manipulation and whose tumours do not show a significant response by 12 weeks are changed to CAP chemotherapy.

Definitive locoregional therapy comprises either i) mastectomy and axillary node clearance or ii) wide local excision, axillary node clearance and post operative radiotherapy. Tumour tissue is taken at surgery for histology, oestrogen receptor assay (where relevant) and further immunohistochemical and biological studies.

Conventional Therapy Arm

All patients in this arm of the trial proceed directly to definitive locoregional therapy as has been described above. Thereafter adjuvant systemic therapy is given as follows:
a) Premenopausal patients with metastatic disease in axillary lymph node(s) are given combination chemotherapy with CMF:

Cyclophosphamide 600mg/m^2 I.V. bolus.

Methotrexate 50mg/m^2 I.V. bolus.

5-Fluorouracil 600mg/m^2 I.V. bolus.

Six cycles of treatment are given three-weekly.

b) All other patients commence tamoxifen, 20mg per day after surgery.

Follow Up

All patients are followed up regularly after surgery and disease recurrences and deaths recorded.

2.3 Monitoring the Response to Primary Systemic Therapy

The principles of primary systemic therapy for operable tumours arose from approaches developed for the medical treatment of locally advanced inoperable disease, as has been discussed. The observed response to chemotherapy of advanced tumours has been reported to be of prognostic significance (Valagussa, 1990) and the same appears to be applicable to operable tumours although follow up data of the initial studies is still relatively short (Bonadonna, 1993). The debate continues, however, as to the most appropriate method of assessment of response. The reported methods in trials of primary chemotherapy have comprised serial calliper measurements and usually additional mammography. According to U.I.C.C. guidelines, data from calliper measurements allow the observed response to be classified as either a "complete response", "partial response", "no change" or "progressive disease" (Hayward, 1977). Mammographic measurements may be expressed similarly. Forrest et al., reporting on the pilot series in Edinburgh, found an excellent correlation between mammographic and clinical response (Forrest, 1986). Others have demonstrated difficulties in quantifying the radiological response and that in a substantial proportion of cases mammography lags behind clinical response to both endocrine and chemotherapy (Moskovic, 1993). An explanation for the discrepancy may be related to the underlying pathological changes with treatment. Hyalinisation or a fibrous reaction within the tumour may alter the clinically palpable mass but not the mammographic appearance. Indeed it is the pathological assessment of response which appears to be of most prognostic significance in locally advanved disease (Feldman, 1986). Interestingly Feldman found that the absence of macroscopic disease in the mastectomy specimen was a significant predictor for disease-free and overall survival and that the absence of microscopic disease did not affect the relationship. Ultrasound examination and measurement of breast tumours has been shown to correlate with pathological examination more closely than mammography (Fornage, 1987) and has proved useful in assessment of response to primary therapy in the Edinburgh study (Forouhi, 1994). Ultrasound has the advantage of being painless and free from potential long term side effects and therefore allows frequent serial assessments to accurately follow the response. However there are still occasional tumours which will fibrose or hyalinise in response to therapy without a significant change in their ultrasonographic appearance. Duplex colour Doppler ultrasound has the advantage of allowing both the standard measurement of the tumour and its blood flow. Most malignant tumours are characterised by increased vascularity and an abnormal vessel pattern. Doppler detection of this vascularity has proved sensitive and specific in the

detection of breast cancer (Britton, 1990) and its serial quantitation, in a recent case report, potentially useful in the assessment response to primary chemotherapy (Madjar, 1993). Although the cost of the hardware at present limits its use it is likely that in the not too distant future a combination of serial Doppler measurements and pre and post treatment mammography will comprise the most useful form of assessment.

Chapter 3 : The Elderly Patient With Breast Cancer

3.1 Historical Aspects

The incidence of breast cancer increases with age and indeed approximately a third of all cases occur in those over the age of 70. However many other medical disorders follow a similar pattern and with a decreasing life expectancy this group of patients present particular problems when considering treatment protocols. Patients over the age of 65 are excluded from most national screening programmes and clinical therapeutic trials. One might expect therefore that patients in this group would generally present with relatively large tumours that would merit a combined therapeutic approach, had they been younger, but with little trial data to support this. A recent review of evidence of determinants of the choice of therapy in the elderly quotes concurrent additional medical complaints or "comorbidity" as a main factor guiding decisions (Satarino, 1992). Two or more conditions (such as arthritis, cerebrovascular and heart disease, cancer and diabetes) are reported by 61% of women aged between 70 and 79 and 70% aged 80 years and older. Satarino cites a study which indicates that of breast cancer patients over the age of 50, 58.9% with "moderate to severe comorbidity" received appropriate therapy compared with 81.3% with little or no comorbidity. However even among patients with little or no comorbidity and stage 1 or 2 disease only 83% of patients aged 70 or over received "appropriate treatment" compared to 95.6% of those aged between 50 and 69. It maybe therefore that chronological age is used, to the detriment of many older women as a proxy for general health and suitability for particular treatments. A similar conclusion was reached in an Australian study showing that aggressiveness of local treatment was inversely related to age in a group of patients over 70 but overall survival was similar, although those with less aggressive local therapy had a greater incidence of local recurrence often requiring a second procedure (Hainsworth, 1992). However, it is also clear that there are many in this age group who obviously could not withstand aggressive treatment or refuse such therapies.

Tamoxifen has been shown, in many studies, to significantly increase disease-free and overall survival when used as adjuvant therapy in patients with breast cancer over the age of 50 (EBCTCG, 1992). Similar results have been obtained in a single-centre study in patients over 65 years of age (Cummings, 1993). This may not entirely be due to the effects of tamoxifen on breast cancer as there appears to be a substantial reduction in deaths due to other causes in those taking the drug, principally from cardiac disease (McDonald, 1991; Rutqvist, 1993). In several trials therapy has been

continued for up to five years with remarkably few significant side effects reported. It is the combination of effectiveness and low toxicity that has prompted several studies testing tamoxifen as sole therapy in the elderly patient. Data from such studies demonstrate that overall approximately 60% of tumours will regress and a further 20% remain static. A summary of results is shown below (Table 3.1)

Table 3.1 Review of Reported Results of Primary Tamoxifen Therapy for Operable Disease in Elderly Patients (Forrest, 1991)

		Response to Tamoxifen			
Trial	No. of Patients	Complete Response	Partial Response	Stasis	Progression
Preece (1982)	67	18	14	17	18
Helleberg (1982)	27	15	5	2	5
Bradbeer & Kyngdon (1983)	161	44	55	39	23
Allan (1985)	53	29	14	6	4
Falk (1989)	43	14	19	-	10
McDonald (1990)	58	10	10	20	18
Robertson (1988)	57	22	9	9	17
TOTAL	466	152	126	93	95
%	100	32.6	27.0	20.0	20.4

Although these results appear encouraging, several studies have described a high rate of relapse, particularly of local disease. An initial trial in Nottingham comparing surgery with primary tamoxifen in a group of patients over the age of 70 with operable disease but no other selection criteria, found no significant difference in overall survival or cause of death between the two groups after five years (Robertson, 1992). However after a mean follow-up of 65 months the majority of patients treated with tamoxifen alone required a mastectomy for local relapse. The conclusion to be drawn from such data is therefore that all elderly patients with operable disease and who are fit should have surgery at presentation to avoid simply postponing an operative procedure in a population in which a delay may be undesirable. However in one-third of the patients, tamoxifen achieved control of disease for up to five years. Patients themselves often wish to avoid surgery, if at all possible, and other selection factors

may identify this subgroup who could be controlled in the long-term by tamoxifen alone. The same group at Nottingham using oestrogen receptor status to select patients for tamoxifen therapy demonstrated, after short-term follow-up, a much lower incidence of early progression in oestrogen receptor positive tumours compared to the unselected tumours of the earlier trial (Low, 1992). However with longer follow-up it seems that even using this criteria disease control fails in approximately 60% of patients after five years (Horobin, 1991). A study performed in Edinburgh aiming to determine predictors of "worthwhile response" to primary tamoxifen in the elderly confirmed the contribution of oestrogen receptor status but failed to demonstrate an association with the early observed clinical response (Gaskell, 1992). Several other predictive factors have been proposed including tumour cell expression of additional steroid receptors, oestrogen inducible proteins and growth factor receptors but the clinical relevance of the published data is inconclusive at present.

There seems little doubt that there is a group of patients who can be maintained on tamoxifen alone in the long-term. One of the aims of this thesis is to attempt to identify this group by means of a combination of clinical observation of response patterns and of examination of underlying tumour biological characteristics and their response to treatment. This approach may also identify patients with very aggressive disease who will require extensive surgery and additional treatment such as radiotherapy to effect good control.

3.2 The Edinburgh Trial of Surgery and Tamoxifen versus Tamoxifen alone for Primary Breast Cancer in Patients Over the age of 70 Years.

In an attempt to further the investigation of the role of tamoxifen in treatment of the elderly patient a new study was commenced in Edinburgh in January 1992.The principal aim was to determine long-term outcome of patients who had initially responded to tamoxifen and had then been randomised to receive either locoregional surgery or a continuation of primary therapy. The protocol is diagrammatically represented below (Fig 3.1). The main inclusion and exclusion criteria are listed as follows:

INCLUSION

a) Patients over 70 years of age.
b) Potentially operable disease (including small T4 lesions).
c) No evidence of metastatic disease.
d) Fit for general anaesthesia.
e) Oestrogen receptor positive tumour (>10% cells staining with E.R.I.C.A. or >20 fmol/mg cytosol protein by dextran-charcoal saturation method).

EXCLUSION

a) Previous malignancy (other than basal cell or squamous carcinoma of the skin or in-situ carcinoma of cervix).
b) Tumour not assessable by ultrasound scanning.

Incorporated within the study are two categories of biological assessment of tumour characteristics. The first aims to compare the two most commonly used methods of estimation of tumour oestrogen receptor expression; E.R.I.C.A. performed on material from fine needle aspiration and the dextran-charcoal saturation method using tissue obtained by open biopsy. These measurements can then be related to the subsequent response to tamoxifen. Secondly the examination of tissue obtained before treatment at the time of wedge biopsy can be compared to tissue obtained after treatment from those who have not responded or those who have responded but have been randomised to receive surgery. This enables biological characteristics to be related to clinical response and may give further information into therapeutic mechanisms of tamoxifen and of tumour resistance. Only those patients with tumours greater than three centimetres in clinical diameter and who agree to the procedure undergo local anaesthetic biopsy.
All patients within the trial receive 20mg tamoxifen daily for an initial period of three

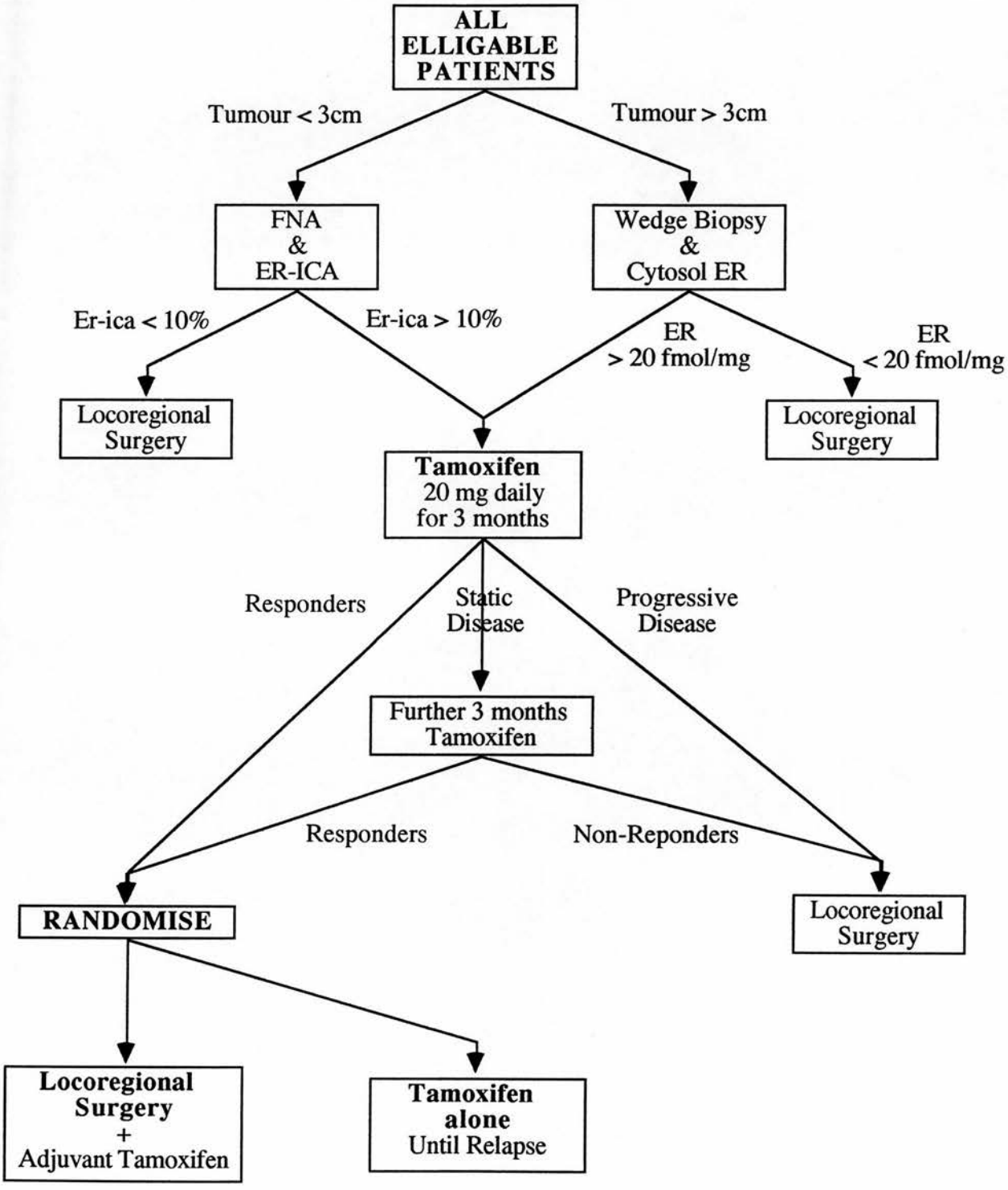
months. The tumours are measured clinically and by ultrasound prior to commencement of treatment (after wedge biopsy, in those undergoing this procedure) and thereafter at four-weekly intervals. All patients are then reviewed at three months. Those who have responded are randomised either to receive definitive locoregional surgery or to continue tamoxifen. Those whose tumours have remained static continue therapy for a further three months and if a response is observed they too are randomised. All patients with progression of disease within the first three months or static disease after six months undergo surgery.

All patients are under the care of one consultant surgeon and the form of locoregional surgery performed is at his disgression. In responding patients a benefit of this approach to tamoxifen therapy is very often a downgrading of the degree of surgery required, so that many can be treated by a local excision of the tumour with conservation of the breast, thus avoiding a full mastectomy. Tamoxifen is continued postoperatively as adjuvant therapy.

Tamoxifen is continued in those patients randomised to receive it as sole therapy until relapse. If the recurrent disease is purely locoregional patients will undergo surgery but if metastatic at distant sites then second line hormonal therapy is given.

All patients are followed up at regular intervals to assess the state of their disease and recurrences and deaths are recorded.

Fig 3.1 Trial Design of the Edinburgh Trial of Surgery and Tamoxifen versus Tamoxifen alone for Primary Breast Cancer in Patients Over the age of 70 Years.



3.3 Monitoring the Clinical Response to Treatment in the Elderly

All studies reported to date have used calliper measurements to assess the clinical response to tamoxifen in elderly patients. Indeed the previous study conducted in Edinburgh analysed the results of multiple measurements over the initial three months of treatment as a possible predictor of long term outcome (Gaskell, 1992). The results indicated this to be unsatisfactory but the authors felt a more accurate method of assessment may improve the predictive value, for outcome, of such measurements. For reasons alluded to previously, serial ultrasound scanning, alone or in combination with more traditional methods of assessment, may prove helpful.

Chapter 4 : Therapeutic Mechanisms

4.1 Adriamycin Based Chemotherapy Regimes

The anthracycline, Adriamycin (or doxorubicin) was first isolated in 1969 from a chemically mutated strain of*Streptomyces peucetius* var. *caesius* (Powis, 1987). Since introduced into clinical practice in the early 1970's it has proved effective against a wide range of both haematological and solid tumour malignancies. Toxicity associated with all the anthracyclines includes myelosuppression, nausea, vomiting and alopecia in the acute phase with cardiomyopathy as a dose-limiting cumulative effect. The precise mode of action of the anthracyclines at a cellular level remains unclear although at least three mechanisms have been proposed; i) High affinity binding to DNA, blocking further DNA, RNA and protein synthesis, ii) alteration of cell membrane fluidity and ion permeability and iii) the generation of free radicals (Powis, 1987). Cell cycle analysis by flow cytometry has demonstrated both *in vitro* and *in vivo* an accumulation of cells in the G_2M region of DNA histograms of tumour cells exposed to adriamycin (Engelholm, 1983; Remvikos, 1993a). This effect can be distinguished from the effects of, for example, alkylating agents such as cyclophosphamide, which provoke an accumulation within the S-phase of the cycle (Engelholm, 1986). Investigation into the mechanisms of intrinsic and acquired cellular resistance to adriamycin have recently centered around the expression of multidrug resistance (MDR) genes. As the term suggests these genes, if activated, can confer resistance, in most malignancies including breast cancer, to several lipophilic cytotoxic agents. The *mdr* -1 gene has been extensively investigated and codes for membrane *P* -glycoprotein, an active efflux pump that can maintain intracellular concentrations of chemotherapeutic agents at nontoxic levels (Ro, 1990).

The accepted approach to chemotherapy for breast cancer involves the use of a combination of different agents. The ratonale behind such practice can be justified firstly by the need to reduce the likelihood of the development of resistance; secondly, by the need to reduce the severity of toxic effects; thirdly, by the hope of increasing the therapeutic effect by synergism or interaction with different aspects of the cell cycle. Following this principle the Edinburgh study of primary systemic chemotherapy employs a combination of adriamycin and cyclophosphamide. Cyclophosphamide is an alkylating agent and exerts its cytotoxicity by inducing DNA strand cross linkage. As mentioned above it appears to affect cells at a different point in the cell cycle than does adriamycin, and does not produce the same cumulative cardiotoxicity. The alkylating agents are potent suppressors of ovarian function and the significance of this

possible contribution to the responses seen in breast cancer remains a topic for debate (Pourquier, 1993; Toma, 1992). Several possible resistance mechanisms to cyclophosphamide are known including MDR gene expression. This would theoretically be contrary to one of the indications of multidrug therapy, but the clinical significance of an apparent common mode of resistance to this drug combination remains to be demonstrated.

4.2 Tamoxifen and Hormone Manipulation

Tamoxifen is a non-steroidal anti-oestrogen, initially found to have antifertility properties in rats and mice (Harper, 1967) and developed as a possible contraceptive agent in man. Its potential clinical use in the treatment of advanced breast cancer was soon identified (Cole, 1971) and its use has become increasingly widespread as both a primary and an adjuvant therapeutic agent. The low incidence of attendant side effects has even led to ongoing trials of tamoxifen as preventative therapy in women with a perceived high risk of developing breast cancer (Powles, 1989).

The mechanism of action of tamoxifen is complex but is thought primarily to be mediated through competitive inhibition of oestrogen binding at the oestrogen receptor site, within those cancer cells in which it is expressed (Jordan, 1993). This inhibitory action decreases cellular proliferation by direct and indirect pathways with effects on gene transcription including reduced production of growth factors (Noguchi, 1993). This theory is borne out in clinical practice with significant responses to primary therapy seen only in cancers containing receptors. However with tamoxifen used as adjuvant treatment although the best survival advantage is seen in patients with oestrogen receptor positive tumours a significant improvement is also seen in patients with receptor-poor tumours (EBCTCG, 1992). This observation has encouraged studies of possible mechanisms of action other than via the oestrogen receptor. Two potentially important alternative pathways by which tamoxifen may decrease cellular proliferation are firstly via the inhibition of the production of autocrine growth factors such as insulin-like growth factor (IGF-1), which have been shown to stimulate breast cancer cell growth and are produced by normal tissues (Huyny, 1993) and secondly through stimulation of the production of the inhibitory transforming growth factor (TGFß) by tumour cells and stromal fibroblasts (Butta, 1992).The effects of tamoxifen on cells that do express oestrogen receptors is further complicated by its ability to act both as an antagonist and an agonist. Oestrogenic effects can be demonstrated in breast cancer cell lines by, for example, the induction of oestrogen inducible proteins such as pS2 (Johnson, 1989) and the progesterone receptor (Horwitz, 1978).These agonist effects are manifested clinically, in postmenopausal women, through beneficial protective effects on bone (Love, 1992) and the cardiovascular system (McDonald, 1991), and potentially harmful effects on blood coagulation and the endometrium (Fornander, 1993).

This variety of effects and possible mechanisms of action against breast tumours make the study of response and resistance to tamoxifen treatment extremely complex. Nonetheless such studies are vital not only because tamoxifen is, arguably, the most

potent endocrine agent available against a broad range of breast tumours but also its mechanisms of action may hold the key to development of further curative and preventative treatments.

Tamoxifen has been reported to cause tumour regression in 50-60% of primary oestrogen receptor positive tumours (McGuire, 1975).The mechanisms underlying these observations remain unclear since tamoxifen has classically been described as a cytostatic agent acting through interference with the oestrogen stimulus to proliferation. This effect has been further accurately defined as an induction of cell cycle arrest with an accumulation of cells in the G_0 / G_1 phase (Osborne, 1983; Thomas, 1992). Other studies have also demonstated a decrease in 5-bromo-2-deoxyuridine (BrdU) uptake and in the expression of the proliferation-associated nuclear antigen, Ki-67, in cell lines exposed to tamoxifen (Danova, 1993). However, to achieve tumour regression both a decrease in proliferation and an increase in cell death are required. Cell death can result either from tissue necrosis or from an active mechanism of programmed cell death known as apoptosis. Apoptosis can be induced by a variety of anticancer agents as well as several other toxic cellular insults (Wyllie, 1993) and recently the tamoxifen analogue, toremifene, has been shown to both inhibit growth and induce apoptosis in hormone-sensitive breast cancer cell lines (Warri, 1993).Although oestrogen withdrawal also led to apoptosis in these cell cultures, mitoses still took place (albeit at a slower rate) and thus it is likely to be the combined inhibitory and apoptotic effects of antioestrogens that lead to tumour regression. The mechanism of induction of active cell death remains unclear although earlier work with 4- hydroxytamoxifen, a potent anti-oestrogenic metabolite of tamoxifen, confirmed that this effect could be inhibited by oestrogen and thus occurs via an interaction with the oestrogen receptor (Bardon, 1987). This would be in keeping with the proposed role of apoptosis in normal tissues undergoing both proliferation/differentiation and involution typified by the female breast and reproductive organs in which cyclical changes are under hormonal control (Ferguson, 1981). An improved understanding of the controlling mechanisms underlying these normal processes would allow a greater perception of mechanisms of action of, and importantly resistance to, the antioestrogens in breast cancer. Studies of apoptosis initially in lymphoma but later in various malignancies and normal tissues have identified the importance of one particular gene,*bcl* -2, in the control of this process (Hockenberry, 1990). The *bcl* -2 gene product has been shown to protect against apoptosis induced by a variety of toxic insults including chemotherapeutic agents (Miyashita, 1992) and interestingly is found in, amongst others, normal hormone-responsive tissues such as the ductal epithelium of the breast (Lu, 1993). This gene has been confirmed to be, at least partly, under

oestrogenic control but its rôle in tumour response and resistance to antioestrogens is unknown. Expression of the gene has been associated with hormone resistance in prostatic carcinoma (Colombel, 1993).

Long term follow-up studies have shown that of the 50-60% of patients with oestrogen receptor positive tumours who initially respond to tamoxifen approximately half will have relapsed after 5 years of treatment (Horobin, 1991). Many theories have been proposed for both de-novo and acquired resistance to therapy but there is little hard clinical or experimental evidence of actual resistance mechanisms. Some potential mechanisms are displayed diagrammatically (fig 4.1) and discussed briefly below. These can be roughly divided into four groups; firstly, mechanisms related to tamoxifen levels within tumours lowered either by reduced inta-tumoral accumulation or by altered metabolism; secondly, defects at the level of the oestrogen receptor with a reduction in receptor levels or mutation and loss or alteration of function; thirdly through altered signal transducton with modification of interactions of the receptor complex with transcription factors or oestrogen response elements at the DNA level, negating the effect of the oestrogen receptor complex on cell processes or influencing the balance of oestrogenic/antioestrogenic effects of tamoxifen; fourthly, indirect mechanisms mediated through altered levels of, or responsiveness to, growth factors such as TGF-ß, TGF-a and IGF-1 and expression of surface receptors such as c-*erb*B-2 (Benz, 1992) and the epidermal growth factor receptor (Long, 1992).

Both xenograft and recent clinical data suggest that the intratumoral levels of tamoxifen are lower in tumours that have acquired resistance than in those remaining sensitive (Wiebe, 1993; Johnston, 1993).One might speculate that reduced levels are the result of a cellular efflux pump mechanism such as *P*- glycoprotein coded for by the *mdr* -1 gene. Interestingly tamoxifen has been shown to reverse resistance to several chemotherapeutic agents mediated by MDR, *in vitro* (Ramu, 1984), although clinical trials to test this finding *in vivo* with high dose tamoxifen have been inconclusive (Stuart, 1992). This effect is thought to take place in a competitive fashion (Leonessa, 1994) and it is therefore possible that at therapeutic levels tamoxifen is a substrate for this pump mechanism. Further evidence for the presence of an efflux pump was the demonstration that R-verapamil, an agent known to be capable of reversing MDR mediated drug resistance, was also capable of reversing tamoxifen resistance in a rat mamary carcinoma model (Kellen, 1991a). However the same group had also found, in an earlier study, decreased levels of *P* -glycoprotein in resistant cells (Kellen, 1991b) and a more recent report has demonstrated a failure of induction of tamoxifen resistance with over expression of *mdr* -1 in a transfected MCF-7, hormone sensitive, breast cancer cell line (Clark, 1992). There is, as yet, little clinical evidence for the

association between tamoxifen resistance and *P* -glycoprotein exists although amplification of the *mdr* -1 has been noted on serial tumour samples obtained by fine needle aspiration during tamoxifen therapy (Lonn, 1992). Decreased intratumoral tamoxifen levels not only allows less competitive inhibition with oestrogen for oestrogen receptor sites but paradoxically may result in stimulation of growth, and another possible mechanism for observed relapse during tamoxifen therapy (Gottardis, 1989). Indeed, in certain instances, there is clinical evidence of a withdrawal response of tumours that have relapsed, after an initial response to tamoxifen, when therapy is discontinued. Lower detectable levels of tamoxifen may not only be the result of an active efflux mechanism but also of intracellular binding to antioestrogen binding sites (Pavlik, 1992) or an altered metabolism with the production of either weakly antioestrogenic or potently oestrogenic metabolites (Morrow, 1993). It would theoretically be possible to observe the balance of agonist/antagonist activity at the oestrogen receptor by observing expression of oestrogen regulated genes in sensitive and resistant tumours.

Detailed descriptions of the other potential mechanisms of tamoxifen resistance are outwith the scope of this thesis but further brief discussion will be found in sections dealing with the particular markers incorporated in this study. If the principal, clinically relevant, mechanisms of resistance to tamoxifen are still to be defined then it is obviously very difficult to predict prior to therapy the 40-50% of patients that will be resistant de novo or the additional 25-30% that will relapse during treatment. Conversely, if predictive factors are found then this may aid the detection of new resistance mechanisms or confirm those already proposed.

Chapter 5 : Prognostic and Predictive Indicators in Breast Cancer

5.1 Introduction

The heterogenous nature of breast cancer, particularly with respect to clinical natural history and response to treatment, has understandably generated an enormous literature on the subject of prognostic and predictive factors for the disease. With new and potentially curative drugs or more potent methods of administering established agents the definition of patient subgroups likely to benefit has assumed vital importance. This being especially the case since these forms of treatment often carry a greater potential for toxic side effects. The awareness of the problem has been heightened by a change in the population of new patients with a greater proportion presenting with early stage disease; a result of the extensive screening programmes introduced over the past 10-15 years. A prognostic indicator maybe defined as a factor which, at the time of diagnosis, is able to give information onclinical outcome. A predictive factor is one which may be able to aid selection of patients likely to respond to a specific form or combination of systemic adjuvant (or neoadjuvant) therapy. It is outwith the remit of this thesis to discuss prognostic factors in great detail but several of the classical factors may also be predictive for response to primary systemic therapy. It is possible to classify prognostic factors into three broad groups: i) patient characteristics; ii) tumour characteristics; and iii) response to therapy.

i) Patient age at presentation is an established prognostic indicator and has been described previously. Other factors which may be important include menopausal status, ethnic origin, geographical origin, intercurrent disease, obesity and diet.

ii) Tumour characteristics include the nearly universal TNM staging upon which selection of individual patients for particular therapies is usually based.This classification takes into account tumour size (T), axillary nodal status (N) and the presence of detectable, distant metastases (M). For primary breast cancer, with no evidence of distant metastatic deposits, axillary node status is often the deciding factor in prescription of systemic adjuvant therapy because of the relatively poor survival of patients with lymph node positive disease. However 30% of patients with lymph node negative disease will still relapse (McGuire, 1992), indicating the heterogenous nature even of subgroups of disease, and it is this group particularly which has generated so much work investigating additional prognostic factors (Table 5.1). Other tumour characteristics can be divided into: a) histopathological, including histological type and grade (Aaltomaa, 1992a); b) biological, including the widespread use of the oestrogen receptor as a marker of differentiation and good prognosis (Desforges, 1992) as well

as a predictive indicator for response to hormone therapy (Hawkins, 1980), and can be further classified as shown below (Table 5.1); c) genetic alterations, including possession of the proposed gene responsible for a subpopulation of breast and ovarian carcinomas that are clearly familial, BRCA 1 (Miki, 1994).

McGuire *et al.* (1990) have formulated a useful overview of certain prognostic indicators that are based on tumour characteristics. A critical appraisal is made of the evidence for individual and combined prognostic factors and then a framework is provided for using this information directly to make treatment decisions. With a plethora of proposed prognostic markers, McGuire *et al.* offer sound advice as to the criteria that must be fulfilled for such markers to be useful in clinical practice.

iii) Direct assessment of tumour response to treatment by primary systemic therapy may be prognostic of overall survival (Bonadonna, 1993; Rodier, 1993) and thus select patient subgroups according to response. Those who, for example, have demonstrated a poor clinical response may benefit from more intensive therapy such as high dose chemotherapy with autologous bone marrow transplant or an additional treatment modality such as radiotherapy. Response can also be monitored at a cellular level, and clinical studies such as those conducted in Edinburgh and described above allow tumour tissue to be assessed for a variety of markers before, after and during treatment. Potentially, patients may then be further subgrouped according to a combination of clinical and biological responses and selected for additional or alternative therapies as required.

Table 5.1 Potentially useful prognostic indicators in node-negative breast cancer.

Conventional pathological features
- Tumour size
- Nuclear and histological grades
- Histological types

Receptors and growth factors
- Oestrogen receptor
- Progesterone receptor
- Epidermal growth factor receptor
- c-*erb*B-2 oncogene product
- H-ras oncogene product
- c-myb oncogene product
- Insulin-like growth factor
- Vitamin D receptor

Tumour proliferation rate markers
- Thymidine labelling index
- S-phase fraction by flow cytometry
- Ki-67, Ki-S1 score
- BrdU labelling index

Tumour angiogenesis markers
- Factor VIII-related antigen
- CD-31 antibody

Growth suppressors or antimetastatic genes
- nm23
- p53 tumour suppressor gene

Local invasiveness markers
- Pro-cathepsin D
- Heat shock proteins
- plasminogen activator
- Laminin receptors

Table 5.1 continued

DNA content and genetic alterations

- DNA ploidy by flow cytometry
- Genetic alterations

Miscellaneous

- Haptoglobin-related protein
- pS2 protein

Adapted from (Gasparini, 1993)

5.2 Predictive Indicators of Chemoresponsiveness

At present there are no factors in clinical use able to predict accurately for response to systemic chemotherapy. This may, in part, be due to the relatively few reported trials of primary therapy and also to the relatively small patient numbers within those trials from whom to draw meaningful data. Two early trials reported some conflicting results with respect to the correlation of response with the classical clinical prognostic indicators such as age, menopausal status, tumour size, histological grade and oestrogen receptor status. An Italian study found the degree of response to be inversely proportional to the initial tumour size and an increased frequency of response in oestrogen receptor negative tumours (Bonadonna, 1990). A French study found a similar relationship with initial tumour size but also found positive correlations with histological grade and age, the incidence of complete regression being higher in less differentiated tumours and in older patients (Jacquillat, 1990). The DNA content, or ploidy, of cells in a particular tumour has been proposed as both a prognostic and predictive factor. Aneuploid tumours have been reported to have a worse overall prognosis although in most studies this does not reach independent significance in multivariate analyses of prognostic indicators (Fisher, 1991a; Stanton, 1992). Aneuploid tumours may, however, be more likely to respond to chemotherapy initially, primarily due to an increased rate of proliferation when compared to diploid tumours (Dawson, 1990; Gnant, 1992). In the neoadjuvant setting, Briffod *et al.* (Briffod, 1989) confirmed this, but Bonadonna *et al.* (Bonadonna, 1990) found ploidy to be of no predictive significance.

Most chemotherapeutic agents exert their effects on proliferating cells and one might therefore expect that tumours with a high proportion of cells in the proliferative phase of the cell cycle would demonstrate the best response. Several studies have shown this to be the case (Remvikos, 1993b; Spyratos, 1992). Remvikos *et al.* also showed a very poor prognosis (after 45 months follow-up) for patients with high S-phase fractions, measured prior to neoadjuvant chemotherapy, who failed to respond. In those patients who did respond pre-treatment S-phase fraction did not appear to affect their long-term outcome. However, the value of S-phase measurement remains the subject of ongoing debate particularly concerning its use as a prognostic indicator and despite an enormous number of studies, with large numbers of patients, opinions are divided. It is likely to be more useful in the short term prediction of response to treatment. The physical determination of the proportion of cells in the "S-phase section" of a cell cycle histogram also presents problems both in mathematical analysis, particularly in aneuploid tumours, and because cells that have arrested in S-

phase cannot be distinguished from normal cycling cells. To overcome the latter problem more dynamic methods of estimating proliferation have been developed including the use of 5-bromo-2-deoxyuridine (BrdU) labelling. This thymidine analogue can be incorporated into tumour cells actively synthesising DNA during S-phase either *in vitro* or *in vivo* and visualised with a flourescence-labelled monoclonal antibody by flow cytometry. Very few studies have related BrdU labelling to therapeutic response but one has shown a predictive power similar to that of S-phase (Remvikos, 1993c). A principal intracellular target for anthracyclines has been shown to be the enzyme topoisomerase II, a nuclear enzyme that alters DNA conformation through breaking and rejoining both strands of the DNA backbone (Tewey, 1984). Low levels of this enzyme in a variety of malignant cell lines has been correlated with a relatively poor response to adriamycin (Kim, 1992; Deffie, 1989). Interestingly the gene encoding topoisomerase IIα lies on chromosome 17q close to c-*erb*B-2, the expression of which has also been proposed to predict for chemosensitivity in some studies (Gasparini, 1993), and indeed, co-amplificaton has been demonstrated in breast cancer biopsy specimens (Keith, 1993).

For a systemically administered cytotoxic agent to be effective it must obviously reach the target compartment of the tumour cell in suficient concentrations. An interesting small study has attempted to relate survival following a modified form of primary systemic chemotherapy to tumour vascularity as determined histologically (Protopapa, 1993). For the 26 patients studied those with a lower degree of vascularisation had a shorter overall survival with a possible explanation of reduced drug penetration. A non-invasive estimate of tumour vascularity may be possible through assessment of tumour blood flow by colour flow doppler ultrasound scanning and it would be of interest to test the predictive value of this relatively new technique. One of the most significant predictive indices of chemosensitivity or resistance to emerge in recent years, although still not widely accepted in clinical practice, is the presence of the *mdr* -1 gene product, the 170-kDa membrane *P* -glycoprotein. The presence of this protein has been associated with not only decreased intracellular accumulation of a variety of cytotoxic agents , but also altered intracellular distribution with decreased intranuclear concentration relative to cytoplasmic levels (Schuurhuis, 1993). There is clinical evidence that the expression of *P* - glycoprotein can predict for poor response to systemic chemotherapy in locally advanced disease (Ro, 1990). Indeed, with *in vitro* evidence that the activity of *P* - glycoprotein can be inhibited by several substances, attempts have been made to improve clinical outcomes by combining anthracycline based regimes with agents such as verapamil, cyclosporine, tamoxifen and quinidine. Most of these studies have been non-randomised but one placebo-controlled

randomised trial failed to show any influence on response rate or survival in patients with breast cancer treated with epirubicin plus quinidine (Wishart, 1994). As yet there are no reports of its use in the prediction of response to neoadjuvant therapy. Although the majority of tumours exhibiting the MDR phenotype express *P* -glycoprotein, there are a subgroup which do not and additional efflux pump mechanisms have been proposed. Enzymes such as the glutathione transferases, which detoxify oxygen free radicals generated by cytotoxics such as adriamycin, have also been reported to be present in higher levels in MDR cells (Batist, 1986) and their overexpression has been proposed as a further predictive factor of chemoresistance. It is likely that there are a variety of possible resistance mechanisms confering the MDR phenotype but at present the most common marker, *P* -glycoprotein, deserves further clinical testing of its predictive power as a marker of chemoresistance.

Other potential indicators of chemosensitivity include the expression of heat shock protein (Ciocca, 1992) and the epidermal growth factor receptor (EGF-R) (Gasparini, 1993). Interestingly, adriamycin has been shown to up-regulate the expression of EGF-R in a human tumour cell line. If this line is grown as xenografts in nude mice the combination of adriamycin and anti - EGF-R antibody therapy appears to be "profoundly synergistic" (Surbone, 1993).

It should be remembered that all these potential predictors of response to chemotherapy, have been investigated, almost exclusively, with respect to the primary tumour. Apart from the neoadjuvant setting when systemic therapy may be given to allow surgical conservation of the breast, the principal reason for treatment is the potential presence of micrometatsatic disease. It is obviously impossible to examine effects of treatment on micrometastases and only relatively few studies have compared primary tumour characteristics and response to that of axillary node metastases. Few parameters have been examined but in one study a significant relationship between proliferation, as measured by BrdU uptake, in both primary and nodal disease has been reported (Goodson, 1993). The correlation was not influenced by age, level of hormone receptors, tumour size or number of positive nodes. However, not only may the micometastatic lesions have different biological characteristics but their local environment is likely to differ considerably from that of the primary tumour and this may have important implications for drug penetration.

5.3 Markers of Hormone Responsiveness

The literature concerning potential markers of responsiveness to hormonal therapy is far more extensive than that concerning chemotherapy. As with cytotoxic therapy none of the classical clinical prognostic factors (ie tumour size, lymph node status or histological type) appear to predict for sensitivity to hormonal therapy. At a cellular level, reports are varied but tend to suggest that tumour ploidy does not predict for response (Robertson, 1991; Skoog, 1991). In one study, however, locally advanced tetraploid tumours were reported to be particularly sensitive to tamoxifen therapy (Baildam, 1986). Cell proliferation, as determined by *in vitro* ^{3}H-thymidine labeling, has been related to the response to tamoxifen in a study of 52 patients with advanced, oestrogen receptor positive breast cancer (Paradiso, 1988). A response was achieved in 88% of "fast proliferating" tumours but only in 46% of "slow proliferating" tumours, although the time taken for a response to become evident was longer in the former group. However, in another study, with similar numbers of patients and using the same technique to measure proliferation, the opposite conclusion was reached (Amadori, 1993).

The major predictive indicator of hormone responsiveness has been the presence of oestrogen receptors through which the response to antioestrogen therapy appears to be primarily mediated (McGuire, 1975). Measurement of these receptors is routinely undertaken by most specialist clinical centres by one of three methods. The dextran-coated charcoal (DCC) technique originally introduced in 1970 (Korenman, 1970) is being superceded by the simpler enzyme immunoassay (ER-EIA), for biopsy material, and the immunocytochemical assay (ER-ICA) which can be performed on material obtained from tumour fine needle aspirates. All techniques have been shown to produce results which predict for response to tamoxifen therapy, with 50-60% of receptor positive tumours responding in the short term. However, although there is a high relapse rate ER-ICA has also been shown to be an accurate predictor of response in the long term, when compared to clinical assessment of response over the first three to six months of treatment (Gaskell, 1992). Although oestrogen receptor level is a relatively good predictor of qualitative response to antioestrogen therapy there has been little work to determine the relationship to quantitative response. This could be particularly important in the neoadjuvant setting if an estimate of the likelihood of avoiding mastectomy were required. In an attempt to improve the predictive value of oestrogen receptors various other markers have been proposed. The presence of progesterone receptors in addition to oestrogen receptors has been reported to predict for response in 75-80% of patients (Thorpe, 1988) and independently to predict for

time to treatment failure in patients with metastatic disease (Ravdin, 1992). The expression of the progesterone receptor is under oestrogen regulation (Horwitz, 1978) and its presence may indicate the possession of fully functional oestrogen receptor apparatus by the tumour cell, which in turn influences susceptibility to antioestrogens working via the oestrogen receptor. Tamoxifen has been shown to induce the expression of progesterone receptors through an oestrogenic effect on the oestrogen receptor (Horwitz, 1978) and a small study has shown a positive correlation of short term induction of receptors (over a mean of 13 days) with subsequent clinical response (Howell, 1987).The changes over a longer period of therapy may be somewhat different and there is some evidence that oestrogenic induction might be a very early effect (over days) and antioestrogenic inhibition occurs as a later (over weeks) effect of tamoxifen (Noguchi, 1988). Since the possession of both steroid receptors is still not wholly predictive of response to hormonal therapy, other oestrogen inducible genes, and their protein products, may contribute as markers of intact and functional oestrogen receptors. Principal among these has been the pS2 gene.

pS2 mRNA was first identified by differential screening of a complementary DNA library derived from the oestrogen responsive, human breast cancer cell line, MCF-7 grown in the presence and absence of oestrogen (Masiakowski, 1982). pS2 mRNA has since been demonstrated in various oestrogen responsive, human breast cancer cell lines including ZR 75 and T47D but has not been found in non responsive lines (May, 1988). A summary of ten large studies examining the incidence of pS2 in clinical material, by mRNA detection and immunocytochemistry for the protein product, showed pS2 expression in 57% of oestrogen receptor positive tumours (Pichon, 1993). Interestingly pS2 was also found in 17% of oestrogen receptor negative tumours but the studies summarised displayed a wide range of 0 to 39%. More recent investigations have concluded that with accurate measurement, particularly of the oestrogen receptor, receptors can be detected in only 2-5% of pS2 negative tumours (Koerner, 1992). The gene has been sequenced (Jakowlew, 1984) and codes for an 84 amino acid-long, cysteine rich, secretory protein found, intracellularly, predominantly in the golgi apparatus. The protein is, as yet, of unknown function, but has features similar to several small protein growth factors such as IGF-1 and also close homology to porcine pancreatic spasmolytic polypeptide (Baker, 1988) which has been reported to have growth stimulatory effects on cell cultures (Hoosein, 1989). However, experiments with pS2-specific antisense oligonucleotide have demonstrated that in breast cancer cell lines which express pS2, it is not essential for cell growth (Shiu, 1993). pS2 has been detected in a variety of normal tissues, including breast (Piggot, 1991), and tumours other than of the breast, principally of the gastrointestinal system

but also the ovary and endometrium (Henry, 1991a). The presence of pS2 in moderately high levels in normal stomach epithelium, which is oestrogen receptor negative, is suggestive of alternative mechanisms of control of expression other than via the oestrogen receptor (Rio, 1988). Indeed, although the gene is flanked by oestrogen responsive elements, the elements responsive to epidermal growth factor are also closely assosocated (Nunez, 1989). Induction by epidermal growth factor has been proposed as a possible control mechanism in oestrogen receptor negative breast tumours, particularly as these tend to express epidermal growth factor receptors (Foekens, 1991). However, it is likely that in breast tumours the gene is almost entirely under hormonal control as evidenced by the relatively low rates of expression in oestrogen receptor negative tumours. The potential usefulness of pS2 as an additional predictive factor for response to endocrine therapy comes largely from the suggestion that although under oestrogen control it appears to be regulated via a different mechanism to that controlling the expression of progesterone receptors (Rio, 1987). Indeed, pS2 appears to be of prognostic value particularly in the subgroup of oestrogen and progesterone positive tumours, perhaps indicating a better degree of differentiation with a more completely biologically active oestrogen receptor system (Foekens, 1990). Reports on the clinical value of pS2 expression with respect to the prediction of response to antioestrogen therapy are varied. One large study has examined the relationship between expression of pS2 and the "response" to adjuvant post operative tamoxifen therapy and found it to be more predictive than either oestrogen or progesterone receptors (Predine, 1992), while others found no improvement over steroid receptors (Luqmani, 1993). It is likely, in the adjuvant setting, that survival and effectiveness of tamoxifen may relate to each markers, with those tumours expressing all three belonging to the best prognostic group. Two studies have shown that pS2 expression in the primary tumour may define a subset of oestrogen receptor positive tumours that on relapse are more likely to respond to tamoxifen (Henry, 1991b; Schwartz, 1991). Studies of pS2 and the response to primary tamoxifen are few and have tended to correlate the expression after primary tamoxifen therapy with the prior response rather than testing it as a predictor of subsequent response to treatment (Henry, 1989). In cell lines, under certain conditions, tamoxifen and its metabolites have been reported to induce pS2 expression (Johnson, 1989), and if this phenomenon also occurs *in vivo* one cannot assume post-treatment expression reflects the situation pre-treatment. In contrast to the case with progesterone receptors there is no evidence relating the effect of tamoxifen on pS2 expression *in vivo* with the clinical response achieved. In summary, the rôle of pS2 as a predictive factor in primary antioestrogen therapy of operable tumours

remains to be defined.

Other oestrogen regulated proteins have been proposed as prognostic factors in breast cancer, most notably cathepsin-D. Tumour expression of this precursor of a lysosomal protease does not, however, appear to predict for response to hormonal therapy (Klijn, 1992).

Growth factor receptors and principally the epidermal growth factor receptor (EGF-R) have been studied extensively as both prognostic and predictive factors. EGF-R has been shown to be present in approximately 48% of clinical breast tumours (Foekens, 1991), with an inverse correlation with the presence of oestrogen receptors (Sainsbury, 1985). The receptor belongs to the c-*erb* group and mediates the mitogenic effects of epidermal growth factor and also, perhaps more importantly, TGFα (Derynk, 1986). A significant relationship between EGF-R expression and a high rate of tumour cell proliferation has been noted in clinical breast tumour samples (Nicholson, 1993). The expression of the receptor may well represent the progression of a cell towards oestrogen independence with the ability to respond to autocrine growth factors. Cell line experiments have demonstrated a decline in the expression of oestrogen receptors and an increase in EGF-R as cells become hormone independent and vice versa as cells regain hormone sensitivity (Long, 1992). Similar events have been proposed to occur *in vivo* with a large proportion of tumours relapsing on tamoxifen found to express EGF-R. Expression of EGF-R prior to treatment in locally advanced or metastatic disease has also been shown to be a negative predictive factor for response to primary therapy with antioestrogens (Nicholson, 1993).

Other predictive factors have been proposed including the c-*erb*B-2 proto-oncogene discussed in the next chapter. However, the same argument holds as to that for prediction of response to chemotherapy, namely that a presumption is made, when considering systemic endocrine therapy for primary diseaese that occult micrometastases will have similar characteristics to the primary tumour. Evidence from studies of macroscopic lesions suggest that at least with respect to the expression of steroid receptors this may not be the case (Mori, 1991).

Chapter 6 : Oncogenes and Breast Cancer

6.1 Introduction

Oncogenes are dominantly acting genes which promote cell transformation. The precursors of oncogenes, protooncogenes such as c-*erb*B-2, have a normal cellular function regulating proliferation and differentiation. With inappropriate activation or expression, these (proto)oncogenes can promote cell transformation usually with excessive, uncontrolled, cell proliferation. The mechanisms underlying such alterations in protooncogene function include gene amplification, overexpression of the gene product or altered function of the gene product as a result of mutation or translocation. A second class of genes also frequently found in mutated form in neoplastic cells are the tumour suppressor genes such as p53. This class of genes performs essential rôles in control of the cell cycle and their loss of function by mutation or allelic loss results in transformation through cell cycle dysregulation.
Studies of oncogenes and tumour suppresor genes in breast cancer, particularly in relation to response to systemic therapy, are few and mostly report evidence concerning c-*erb*B-2, p53 and more recently c-*myc*.

6.2 c-*erb*B-2

The c-*erb*B-2, HER-2 or *neu* proto-oncogene was initially described in neonatal rat neuroblastoma , and has since been shown to induce mammary tumours in transgenic mice by a single step process (Muller, 1988). The different terms used to describe this gene are some what interchangeable, *neu* referring to the original rat gene and c-*erb*B-2 and HER-2 reflecting different methods of cloning the human homologue. The gene codes for a 185 kDa transmembrane glycoprotein (p185) which shares homology, particularly in the intacellular domain, with EGF-R. A recent review found c-*erb*B-2 gene amplification in 22% of 5941 patients with breast cancer involved in 39 studies and p185 overexpression in 22% of 10074 cases in 44 studies (Dawkins, 1993). The external domain of the p185 protein has structural similarities to growth factor receptors, and recently several possible ligands have been identified which appear to be quite distinct from the ligands for EGF-R. Stimulation of the receptors with these ligands appears to induce a variety of responses including proliferation and differentiation (Tripathy, 1994). Unlike the situation with the presence EGF-R, there appears to be no, or only a weak, correlation of c-*erb*B-2 expression and a high proliferative rate as measured by various indices including Ki-S1, the proliferation-

associated nuclear antigen (Kreipe, 1993). Interestingly several studies have confirmed this finding in invasive carcinomas but have shown a strong positive correlation between proliferation and c-*erb*B-2 expression in ductal carcinoma *in situ* (O'Reilly, 1991; Poller, 1991). One study has also shown a positive relationship between the two indices in oestrogen receptor positive but not in oestrogen receptor negative disease (Borg, 1990). There have been many reports on the use of c-*erb*B-2 as a prognostic indicator with differing conclusions. Particular problems in interpreting these studies include the relatively small percentage of tumours with evidence of gene activation and the varying methodologies used to evaluate gene amplification, mRNA transcription or oncoprotein expression. Also the observed increased expression in *in situ* disease, with over 70% of lesions showing positivity, suggests that the prognostic significance may be different for different stages of the disease (Allred, 1992a). Most studies do agree that detection of gene amplification/overexpression or especially the p185 oncoprotein does imply a poorer prognosis in a subset of patients with axillary node positive disease (Toikkanen, 1992). The presence of the marker is however not independently highly significant for poor prognosis and these patients are all likely to receive adjuvant therapy as a result of their nodal status. The results of studies in node-negative patients are unfortunately much more conflicting, this being the group of patients for whom indicators of poor prognosis and the need for adjuvant therapy are particularly needed. The expression of c-*erb*B-2 may aid in particular subsets of this population and indeed appears to predict for poor outcome in patients with small, oestrogen receptor positive, node-negative tumours (Allred, 1992b).

There are few studies examining the use of c-*erb*B-2 expression as a predictor for response to systemic therapy. The implication of the poor clinical outcome of patients receiving postoperative adjuvant chemotherapy for tumours overexpressing c-*erb*B-2 (both node-positve and -negative) has been that the gene confers a degree of chemoresistance (Gusterson, 1992; Allred, 1992b).Furthermore antibodies directed against c-*erb*B-2 receptor protein can enhance the cytotoxicity of adriamycin (Tripathy, 1994). However, data from a study of the effects of adriamycin on an MCF-7 cell line transfected with c-*erb*B-2 gene could demonstrate no decrease in sensitivity (Benz, 1992). Interest in the situation regarding the response to hormone therapy has been heightened by reports of an element of oestrogen control in the expression of c-*erb*B-2, with oestrogens inhibiting expression *in vitro* at both mRNA and protein levels (Dati, 1990). Conversely tamoxifen has been shown to increase the expression of c-*erb*B-2 at both these levels in oestrogen responsive cell lines *in vitro* (Antoniotti, 1992). This effect was not found in hormone independent breast cancer cell lines suggesting the effect of tamoxifen is mediated by the oestrogen receptor. There is no

evidence, at present, of similar effects *in vivo* , although one study has shown a decrease in c-*erb*B-2 expression with tamoxifen therapy but predominantly in oestrogen receptor negative tumours (Le Roy, 1991). Indeed, most clinical studies have found an inverse relationship between c-*erb*B-2 and oestrogen receptor expression (Todd, 1992).The study of a c-*erb*B-2 transfected, hormone sensitive, cell line demonstrated the induction of tamoxifen resistance, but retention of oestrogen dependence, both in cell cultures in *vitro*, and in xenografts *in vivo* (Benz, 1992). The rôle of c-*erb*B-2 in the prediction of clinical response to antioestrogens is still a subject of debate, with some studies suggesting that overexpression of the gene predicts for therapeutic resistance (Wright, 1992), while others have found no, or only a weak correlation with response (Skoog, 1992; Nicholson, 1993). It may be difficult to interpret these contrasting results since the first study looked at response in advanced and recurrent carcinoma whilst the others comprised elderly patients treated with primary tamoxifen. Nicholson's study also looked at the relationship between EGF-R and c-*erb*B-2 oncoprotein expression and the response to tamoxifen. Whilst high levels of EGF-R alone predicted lack of response to tamoxifen, in tumours expressing moderate levels, coexpression of c-*erb*B-2 improved predictive value of tamoxifen resistance.The combined expression of these related receptor proteins also appears to select patients who have a particularly poor overall prognosis (Osaki, 1992).

In the light of the evidence presented it might be possible to suggest that c-*erb*B-2 activation may be particularly significant in oestogen receptor positive disease in which it demonstrates a breakdown in the normal oestrogen mediated suppression of gene expression. This breakdown of hormonal control may be important in the development of antioestrogen resistance. In a similar fashion to EGF-R, c-*erb*B-2 may then mediate signals resulting in increased cellular proliferation with the effects of the two markers being additive in the small subset of tumours in which they are coexpressed. Other markers have been looked at in conjunction with c-*erb*B-2 in an attempt to improve both the understanding of its mechanism of action and its prognostic and predictive value. Such markers include the tumour suppressor gene p53, the protein accumulation of which has been positively correlated with c-*erb*B-2 expression (Lipponen, 1993a).

p53

The p53 gene protein is a nuclear phosphoprotein originally identfied in extracts of transformed cells reacting with antiserum from animals innoculated with tumour cell lines transformed by simian virus 40 (Lane, 1979). The p53 protein is thought to act, in all normal cells, as a negative regulator of the cell cycle, inhibiting progression of cells from the G_1 to the S-phase of the cycle (Levine, 1991). It is therefore the loss of function, through a variety of mutations of the gene, which is the tumorigenic step. Indeed, transfection of the "wild-type" p53 gene into the T47D breast carcinoma cell line, known to contain a mutant p53 gene, has been shown to lead to a failure of cell growth (Casey, 1991). p53 mutations appear to be the most common genetic alteratons in all human cancers. The gene has been postulated as the target for various environmental carcinogens/mutagens such as aflatoxin and hepatitis B virus in hepatocellular carcinoma, UV light in skin cancers, and tobacco and alcohol in lung and oesophageal cancers (Coles, 1992). The gene has been localised to the short arm of chromosome 17 and allelic loss in this area has been noted in appproximately 60% of unselected breast cancers (Mackay, 1988). The abnormal p53 protein produced as a result of genetic mutation is more stable than the wild-type protein, having a much longer half-life. This allows detection of the mutant protein by immunohistochemistry, a technique very difficult to employ in detecting the very short-lived wild-type protein. However, there is some evidence that altered or abnormal p53 protein degradation may allow wild-type protein to be detected in some circumstances (Wynford-Thomas, 1992). Immunohistochemical studies have shown nuclear p53 protein (as detected by the pAb1801 antibody which detects mutant and wild-type p53 protein) to be present in approximately 40-50% of breast cancers (Silvestrini, 1993; Bosari, 1992; Poller, 1992). Attempts to correlate p53 expression with other histopathological indices have produced varying results. Most studies have shown a positive correlation with increasing histological grade (Lipponen, 1993b) and a negative correlation with the expression of oestrogen receptors. In view of the proposed mechanism of tumorigenesis resulting from loss of p53 function, a relationship between the detection of p53 protein and cell proliferation would be expected. A positive correlation between proliferation, as measured by both flow cytometric S-phase fraction and expression of the proliferation associated antigen Ki67, and abnormal p53 expression has been demonstrated (Lipponen, 1993a; Allred, 1993).

The rôle of p53 as an independent prognostic or predictive factor remains under discussion. Some authors demonstrate independent prognostic value for decreased relapse-free and overall survival (Elledge, 1993) whilst others have found no

independent value when S-phase fraction is included in multivariate analysis (Isola, 1992). There is very little evidence of the place of p53 in prediction of tumour response to systemic therapy. It might be postulated that if p53 in normal cells is associated with close regulation of the cell cycle allowing time for DNA repair, should damage or mistakes in synthesis occur, response to DNA damaging cytotoxic agents would be enhanced. An effect exacerbated by the increased proliferative rate associated with abnormal p53 expression. Few data are available to test this hypothesis, but one small study has shown no correlation of abnormal p53 expression, in 15 primary tumours, with the response of respective recurrent disease to an adriamycin containing chemotherapy regimen (Cantwell, 1992). With respect to the prediction of chemoresistance it has been shown that expression of the *mdr* -1 gene may be associated with some p53 mutations (Chin, 1992). Furthermore, there is evidence that wild-type p53 represses the *P* -glycoprotein gene promoter, whilst mutant forms enhance its activity (Zastawny, 1993). There appears to be no data relating p53 to hormone therapy other than the inverse correlation with the expression of oestrogen receptors.

Chapter 7 : Clinical and Laboratory Techniques Employed in the Prediction and Assessment of Tumour Response

7.1 Fine Needle Aspiration

The technique of fine needle aspiration to obtain material from tumours for cytological examination was introduced in the 1930's but it was not until the 1950's that the practice became widely accepted (Lever, 1985). By 1968 a Swedish group were able to confirm the clinical diagnostic value of the technique by reporting on 3,479 consecutive aspirations from breast masses (Franzen, 1968). Fine needle aspiration cytology was introduced into the Edinburgh Breast Clinic in 1977. One of the important findings of reviews of early experience in Edinburgh was the importance of a single operator in producing diagnostic sensitivity of 99 per cent with a specificity of 95 per cent (Dixon, 1984a). This advice has been followed in the work reported in this thesis with all aspirates (other than those performed intra-operatively) performed by the author after gaining experience as the sole "aspirator" in the new patient breast clinics. The technique used also followed the established method in Edinburgh.

Fine needle aspiration is uncomfortable for the patient, but usually not unduly painful, and has few side effects, with the most frequent being local bruising and occasional haematoma formation. The possibility of local needle track seeding of tumour cells or even of distal dissemination via blood or lymphatics has been raised periodically since the introduction of the technique. An early American study, however, could find no difference in overall 15 year survival rates between groups of patients who had fine needle aspirations of their breast tumours and those who did not (Berg, 1962).

Although fine needle aspiration remains predominantly a diagnostic tool, its potential in providing material for immunocytochemical, and more recently flow cytometric analysis has been recognised. It is particularly suitable for monitoring cellular changes during treatment as sequential aspirations can be performed with a minimum of disturbance to the tumour. A problem with serial aspirations of the same tumour is, however, the potential for intra-tumoural heterogeneity and a resulting difficulty in making comparisons of, for example, antigen expression or cell proliferation (Mullen, 1989). The chances of such a problem can be reduced by passage of the needle through different quadrants of the tumour during aspiration.

7.2 Flow Cytometry

Flow cytometry is potentially the ideal technique for analysing the small amount of material obtained by fine needle aspiration. Flow cytometers were initially developed in the 1960's principally for the analysis of peripheral blood samples. The instrument allows rapid and precise analysis of many thousands of individual cells, processed one by one, in only a few seconds. By laser excitation of fluorochrome-labelled monoclonal antibody and detection of resultant fluorescence a quantitive estimation of bound antibody and thus antigen expression can be made. With an extensive range of monoclonal antibodies against leucocyte antigens now available, haematology departments tend to rely heavily on this technology. The application of the technique to the analysis of solid tumours depended on the development of reliable methods of tissue disaggregation and preparation of single cell suspensions. Although analysis of membrane-bound, cytoplasmic and nuclear antigens has been made possible with the development of the appropriate monoclonal antibodies, it has been the analysis of DNA content and cell cycle kinetic parameters that has been most reported. DNA-specific fluorochromes, such as propidium iodide, are made to fluoresce during passage of cells through the flow cytometer. The resulting level of fluorescence recorded for each cell is a measure of its DNA content. Cells in the $G_{0/1}$-phase of the cycle have a constant DNA content and form a peak in the cell cycle DNA distribution. Cells in the G_2/M-phase also have constant DNA content but twice that of the $G_{0/1}$-phase and form a second peak in the distribution histogram. S-phase cells are undergoing accumulation of new DNA and form a continuum between the two peaks of the histogram (Fig 10.1). Proportions of cells within each of the regions of the histogram can then be calculated. A much debated limitation of such single-parameter DNA analysis of tumour cell populations is the inability to distinguish diploid tumour cells from normal infiltrating leukocytes or stromal cells. Aneuploid tumour cells by definition have an abnormal DNA content and can be identifiedby the appearance of a seperate set of peaks from those produced by normal cells.

Developments in flow cytometers now allow detection of fluorescence in two or three wavelength bands simultaneously. Therefore, labelling different monoclonal antibodies with different fluorochromes potentially allows the expression of two or three antigens to be measured simultaneously on the same cell. This may not only allow identification and exclusion of normal cells from diploid tumour DNA analyses but also enables the relationships between the expression of different antigens on individual cells to be studied. It also increases the number of analyses that can be performed on small amounts of tissue, such as that obtained from fine needle

aspiration. Flow cytometry, however, at present remains purely a research tool in the study of solid tumours, with its obvious potential not yet fully realised.

7.3 Immunohistochemistry

The technique of immunohistochemistry is now firmly established as both a diagnostic and research tool. In common with flow cytometery it has relied upon advances in monoclonal antibody technology with the basic methods being unchanged since its inception. There have, however, been some refinements in methodology principally directed at the improved exposure of particular antigens to the antibodies. These include the treatment of fixed tissue sections with both a variety of enzymes and, more recently, microwave heating.

The great advantage of immunohistochemical techniques over, for example, flow cytometry, is the preservation of tissue architecture allowing spatial localisation of antigen expression as well as an idea of the hetero/homogeneity of expression within the sample taken. The disadvantages of the technique, however, include the length of time taken both in processing and interpreting antibody staining and the difficulty in producing a reproducible quantative estimate of antigen expression. There is also some concern over the use, particularly in breast tumours, of one tissue section as representative of the tumour as a whole.

7.4 Animal Models

Since the initial observation made in the late 1960's that human colon adenocarcinoma grew progressively in an athymic mutant strain of mouse (Rygaard, 1969), such "nude" mice have been frequently used in cancer research. Generally the histological and biochemical properties of tumours grown as xenografts are said to closely resemble those of the original tumour specimens (Price, 1990). However, different types of carcinoma vary in their abilities to grow in nude mice and primary breast tumours have proved particularly difficult to establish as xenografts, with reported "take rates" of only 6 - 15% (Mehta, 1993). Although primary tumours have, so far, proved difficult to grow as xenografts, there are several human breast cancer cell lines, that have been established by tissue culture techniques, which have proved much easier to grow as xenografts in the nude mouse. Such cell line xenografts may have many different properties to primary tumours in the human breast but they will also share many similarities and may be useful tools in the study of biological therapeutic mechanisms. As part of the work for this thesis cell line xenografts have been used to establish methodology before examining the biological responses to systemic therapy in patients undergoing primary systemic therapy.

Because of the potential problems of working with cell line xenografts more work continues into improving xenografting techniques for primary breast tumours. Pilot work performed in the department suggested that coimplantation of a mixture of Matrigel (itself a mixture of laminin, collagen IV, heparan sulphate, and enactin), fibroblasts and tumour cells could enhance the take rate of a cell line found to be particularly difficult to grow as a xenograft (Professor P. Forrest, Personal Communication). The particular contribution of Matrigel to improved xenograft growth appears to have been since confirmed with tissue from primary breast tumours and from a variety of other solid tumours (Mehta, 1993). A reliable method for establishing individual tumours as xenografts would aid tremendously in tailoring systemic treatment to particular tumours by providing a therapeutic test-bed.

Chapter 8 : The Aims of the Thesis

The work leading to the compilation of this thesis set out to characterise the response of primary breast carcinomas to primary systemic therapy. Monitoring of the macroscopic response to treatment by the use of serial ultrasound scanning had been successfully employed within the Edinburgh Breast Unit for assessing response to chemotherapy and to a lesser extent hormonal therapy. The majority of patients studied for this thesis were treated, under the protocol for elderly patients, with primary tamoxifen. This gave an opportunity to characterise macroscopic responses to tamoxifen by an accurate means of measurement, being data not, so far, reported.

Having an accurate assessment of the macroscopic response to therapy gave an excellent opportunity to examine the corresponding cellular responses. Such data would help to identify not only predictive biological factors for response to individual therapies but also aid understanding of mechanisms of action of, and resistance to, these treatments.

The philosophy adopted in attempting to examine these biological responses has been to develop methodologies initially in human breast cancer cell line tissue culture systems. To then apply some of the the same techniques to material obtained from cell line xenografts and finally to tumour tissue obtained from patients with breast cancer.

Verifying the applicability of techniques to xenograft material was also important since a long-term plan to follow on from this work is to develop a reliable method of xenografting primary breast tumour material. If this becomes possible then testing susceptibility of individual tumours, as xenografts, to a variety of agents would significantly aid clinical management and, being more accessible, would allow even closer observation of biological tumour responses to systemic therapy.

The immediate aims of the thesis can therefore be summarised as:

i) to further sharacterise the clinical response to primary systemic therapy of breast cancer in the setting of two clinical trials,

ii) to determine whether it is possible to identify biological markers in pre-treatment tumour specimens that predict for subsequent response to therapy,

iii) to determine the effects of treatment on these biological markers and whether changes relate to the clinical response,

iv) to identify markers of resistance.

MATERIALS and METHODS

Chapter 9 : Methods

9.1 Patient Recruitment

All patients studied presented to the Edinburgh Breast Unit, situated initially at Longmore Hospital and latterly at the Western General Hospital in Edinburgh. Patients were individually counselled after diagnosis and staging investigations were completed and their suitability for either the "Primary Systemic Therapy" or the "Elderly Patient" had been discussed by a multidisciplinary clinical team. Informed consent was obtained from each patient and then those entered into the "Primary Systemic Therapy" trial were randomised by the Scottish Cancer Trials Office. All clinical follow-up was performed by the author for the length of the study period. Operative procedures were performed under the authority of either Mr. U. Chetty or Mr. J.M. Dixon, consultant surgeons in the Edinburgh Breast Unit.

9.2 Ultrasound Monitoring of Tumour Size

Patients within the Primary Systemic Therapy trial received either three-weekly cycles of C.A.P. chemotherapy or hormonal therapy with either continuous tamoxifen for postmenopausal patients or four-weekly LHRH agonist goserelin for premenopausal patients. Patients were reviewed on a weekly basis to monitor symptoms and perform ultrasound measurements. Ultrasounds were performed using a Siemens SL1 machine (Siemens, Tokyo, Japan) with a 7.5-MHz linear array probe. Patients within the elderly patient trial were seen for review and for tumour ultrasound measurements on a four-weekly basis.

The probe was held at a right angle to the skin surface and moved over the tumour until a maximum diameter was visualised. Four measurements were made at 45^0 intervals, and the mean diameter and depth of the tumour measured with the machine's electronic callipers. The tumour volume was calculated using the following formula, a method recently reported from the Edinburgh Breast Unit (Forhoui, 1994):

$$\text{Tumour Volume} = \frac{D^2 \times d \times \pi}{6}$$

(Where "D" = mean of diameters parallel to skin surface and "d" = those taken at 90^0). Follow-up serial volumes were expressed as percentages of the initial volume, which was measured, where possible, after wedge biopsy and certainly prior to commencement of therapy.

9.3 Procurement of Tissue Specimens

Tissue was obtained from each patient from the wedge biopsy prior to treatment and from the mastectomy or wide local excision after primary therapy. With the help of Dr. T. Anderson and colleagues in the Department of Pathology at the Western General Hospital, fresh tumour material was obtained after specimen sectioning as soon as possible after removal from the patient (an average time of approximately 20 minutes). Tissue was immediately stored and then maintained in liquid nitrogen.

Fine needle aspirates were performed using a 21 gauge needle attached to a 10ml syringe. Aspirates of the primary tumours *in situ* were taken before treatment by the surgeon performing the wedge biopsy, and by the author in the outpatient clinic at serial time-points during therapy. The same technique was employed to obtain post treatment samples but from the surgical specimens obtained from mastectomies or wide local excisions. Aspirates were taken by passage of the needle through four quadrants of the tumour. The material obtained was divided and expelled either into a storage medium, containing 10% DMSO in foetal calf serum, before freezing and storing in liquid nitrogen, or into an incubation mixture for BrdU incorporation (See section 9.7).

9.4 Preparation of Material from Fine Needle Aspirates

Material obtained from fine needle aspiration was analysed by flow cytometery which requires tissue to be in the form, as much as possible, of a single cell suspension. Material was thawed slowly to room temperature and washed free of storage medium with PBS. To obtain a single cell suspension it was usually sufficient to pass the specimen once through a 23 gauge needle. This was done after a fixation step, in the preparation for analysis, to minimise cell damage.

9.5 Preparation of Tissue for Immunohistochemistry

Tissue samples from those specimens obtained prior to and after primary therapy and stored in liquid nitrogen were fixed in 10% formaldehyde. These were then processed to paraffin blocks by the Edinburgh University Department of Pathology. Sections, 3μm thick, were cut, floated and dried onto plain glass slides. Staining for p53 and Bcl-2 required microwaving of sections and for these analyses sections were dried onto lysine-coated slides.

9.6 Flow Cytometry - DNA Analysis

DNA analyses were performed by flow cytometry using a FACScan cytometer (Becton Dickinson Immuncytometry Systems, Mountain View, California) with a 488nm argon laser for excitation.. Cells obtained from tissue culture of human breast cancer cell lines or material obtained from fine needle aspirates of clinical primary breast tumours were analysed to determine ploidy and cell cycle distribution. Approximately 2 x 10^6 cells were fixed in 70% ethanol, washed and then resuspended in 1ml of PBS. The sample was then stained by the addition 100μl of a .01% solution of propidium iodide (Sigma) in PBS for 20 minutes in the dark at room temperature. The samples were then run through the FACScan collecting 10,000 events, at approximately 75 per second, with CellFIT software (Becton Dickinson). After gating on forward versus side scatter and red fluorescence area versus width dot plots, DNA histograms were analysed with the "SFIT" computer model.

Histograms were deemed uninterpretable if the cefficient of variation of tumour G_0/G_1 peaks exceeded 5%. In clinical specimens, tumour ploidy was defined as either DNA diploid in the presence of one G_0/G_1 peak or aneuploid with two peaks and a ratio of tumour to non malignant cell G_0/G_1 peaks (DNA Index) of greater than 1.1. The DNA diploid peak in an aneuploid histogram is attributed to normal breast cells, non-DNA aneuploid tumour cell clones, tumour lymphocytes and connective tisue cells. The tumours were described as DNA tetraploid if the DNA Index was between 1.95 and 2.05, the number of events in the tumour peak greater than 15% of the total histogram events and a recognisable tumour G_2/M was present, and multiploid if several peaks were seen.

All clinical samples defined as diploid were examined histocytologically, after cytospins had been made, to confirm the presence of tumour cells.

9.7 Flow Cytometry - BrdU Incorporation

Cells obtained from human breast cancer cell lines grown in tissue culture or as xenografts in nude mice or from fine needle aspirates of clinical primary breast tumours were incubated in the presence of BrdU. Cells were suspended in 1ml of RPMI growth medium (Gibco) containing 10% foetal calf serum, 30μmol BrdU and 20μmol Fluorodeoxyuridine (FdU), the latter included to block thymidine kinase activity allowing maximum integration of BrdU. The mixture was incubated for 20-30 minutes at 37^{0}C and then the cells washed and resuspended in RPMI with 10% foetal

calf serum. The mixture was incubated for a further two hours at 37^{0}C and then, after the addition of 10% DMSO, frozen and stored in liquid nitrogen until analysis.
The detection of incorporated BrdU involved DNA denaturation and application of a monoclonal antibody. Stored samples (approximately 2 x 10^6 cells) were thawed and washed free of DMSO before fixing with 70% ethanol on ice for 30 minutes. To ensure maximum nuclear yield and clean histograms samples derived from fine needle aspirates were treated with a solution of pepsin (4mg in 0.1mmol HCL) for 45 minutes at 37^{0}C. DNA denaturation was achieved by a 4N HCL / Triton-X mixture for 30 minutes in the dark. After washing in borax (pH8.5) and then a washing solution comprising PBS, 0.5% Tween-20 and 1% foetal calf serum, 20μl of FITC conjugated anti-BrdU monoclonal antibody (Becton Dickinson) was added for 30 minutes in the dark. After a further wash, samples were DNA counterstained with propidium iodide as described above. Samples were then run through the FACScan and both red and green fluorescent emissions collected for 10,000 events.

9.8 Flow Cytometry - Detection of Ki-67 Antigen

Cell samples were thawed and washed free of storage medium. To approximately 1x10^6 cells 200μl of "membrane shredding solution" was added for 15 minutes at room temperature. After washing 15μl of FITC conjugated monoclonal anti-Ki-67 antibody (Dako) was added for 15 minutes, on ice, in the dark. The samples were then analysed on the flow cytometer collecting green fluorescent emissions and performing analysis with LYSIS II software (Becton-Dickinson).

9.9 Flow Cytometry - Detection of Ki-S1 Antigen

Cell samples were thawed and washed free of storage medium. The cells were fixed in 1ml of methanol at -20^{0}C for 5 minutes. After washing in detergent-based buffer, anti-Ki-S1 monoclonal antibody (2.5μl, Prof. H.Kreipe) was added, and left for 60 minutes at room temperature. After repeat washing in buffer, anti-mouse FITC (100μl of 1:20 dilution, Dako) was added for 30 minutes, on ice, in the dark. After a further wash cells were resuspended in 1ml of PBS and counterstained for DNA with the addition of 100μl of propidium iodide. Red and green fluorescent emissions were collected on the flow cytometer using cellFIT software and analysis was performed using LYSIS II (Ki-S1) and cellFIT (DNA) software.

9.10 Flow Cytometry - Detection of pS2 and c-*erb*B-2 Gene Products

To maximise the information gathered from the relatively small amount of material obtained from fine needle aspirates of clinical tumours, cells were dual-stained for pS2 protein and c-*erb*B-2 protein product. Cell samples were thawed and washed free of storage medium and fixed in 70% ethanol for 30 minutes on ice. After a wash in buffer, anti-pS2 antibody (100μl of a 1:5 dilution, Europath Ltd.),was added and left for 30 minutes on ice. After a further wash, phycoerythrin (PE) conjugated anti-mouse antibody (100μl of a 1:20 dilution, Dako) was added and again left on ice, in the dark, for 30 minutes. Cells were then washed in buffer and anti-c-*erb*B-2 protein antibody, pre-conjugated to FITC label, (10μl, Novocastra) was added and left, on ice, in the dark, for 30 minutes. After a wash in buffer, samples were run through the flow cytometer collecting and analysing both red (pS2) and green (c-*erb* B-2) emissions using LYSIS II software.

9.11 Flow Cytometry - B lymphocyte and Natural Killer Cell Detection

To determine the levels of both B lymphocytes and natural killer cells within fine needle asptirates samples, these were dual stained with antibodies towards CD19 (B cell specific) and CD56 (natural killer cell specific) surface antigens. Samples were thawed and successively washed free of storage medium followed by PBS/5% foetal calf serum buffer. Anti-CD 56 antibody (10μl, Dako) was added and left for 30 minutes on ice. After a wash, FITC conjugate (100μl of 1:20 dilution) was added and left for 30 minutes, on ice, in the dark. After further washing, PE-conjugated anti-CD19 antibody (10μl, Dako) was added and leftr for 30 minutes, on ice, in the dark. The samples were run through the flow cytometer with red and green fluorescence collected and analysed by LYSIS II software. Importantly, cells demonstrating the typical scatter pattern of lymphocytes were included in the gating process on analysis (excluded on analysis of tumour cells).

9.12 Flow Cytometry - *P* -Glycoprotein Detection

Samples were thawed and washed free of storage medium, and then fixed for 5 minutes on ice with 50/50 solution of acetone and methanol. After washing in the standard detergent based wash buffer, 100μl of mouse monoclonal anti-*P* -

glycoprotein antibody, clone JSB-1 (Novocastra), diluted 1:5 was added for 45 minutes on ice. After washing in wash buffer, the samples were fluorescently labelled with FITC as described previously.After further washing and resuspension in PBS, green fluorescent emissions were collected on the flow cytometer and analysed using LYSIS II software.

9.13 Flow Cytometry - Adriamycin Detection

Several Patients receiving adriamycin therapy as part of the "Primary Systemic Therapy Trial" underwent fine needle aspiration of their primary tumours prior to chemotherapy and then again 1hour after completion of the intravenous infusion. These samples were taken at the time of the first and third cycles of chemotherapy.
Stored samples were thawed and washed and resuspended in PBS. Adriamycin is intrinsically fluorescent, emitting in the red range, and therefore with no need of further processing, the samples were run through the flow cytometer collecting red fluorescence. The mean red flourescence of the sample taken prior to adriamycin infusion, acting as a background control, could then be compared to that taken after infusion as an estimate of cellular uptake at 1 hour.

9.14 Immunohistochemistry

The methods used to detect a variety of biological markers by immunohistochemistry were essentially similar, with the exception of the*bcl*- 2 and p53 proteins. The standard method will be described below and antibodies and the dilutions used for individual markers are listed in table 9.1.
For immunohistochemical assessment of tissue samples taken prior to, and after treatment, 3μm sections were cut from paraffin embedded tumour blocks. The sections were de-waxed and then placed in 1% Hydrogen peroxide in methanol to block endogenous peroxidase activity. For some antibodies (see table 9.1), after washing in tap water the sections were treated with distilled water containing 0.1% trypsin and 0.1% calcium chloride corrected to pH7.4 for 30 minutes at 37^{o}C. After successive washes in water and tris buffered saline (TBS) the sections were incubated with 20% foetal calf serum (FCS) to reduce non-specific staining. The sections were then incubated with the respective monoclonal antibody, diluted with 20%FCS/TBS, for 30 minutes at room temperature. Control sections were maintained on 20% FCS. After washes in TBS and incubation at room temperature for 20 minutes with biotinylated rabbit-anti-mouse antibody (Dako) diluted 1:100 in 20%FCS/TBS, staining was

visualised by a standard avidin-biotin-peroxidase method, originally described by Hsu *et al.* (Hsu, 1981). Sections were finally counterstained with haematoxylin.

Immunostaining for both p53 and *bcl* -2 protein is aided by the use of microwave heating to improve antigen retrieval. This treatment required that the sections had been fixed to glass slides using an adhesive, poly-L-lysine. Sections, after dewaxing, were placed in a citrate buffer and then microwaved for 10 minutes at full power in a domestic, 800 watt, microwave oven (Shi, 1991). After microwaving sections were stained as described above.

All sections were scored by an independent single pathologist, with no acess to clinical information. The percentage of tumour cells staining was noted in 10 low power fields and averaged. Sections were then given a score of 0 - 3 representing :

0 = 0 - 5% of tumour cells staining
1 = 5 - 25% " "
2 = 25 - 75% " "
3 = 75 - 100% " "

Table 9.1 Antibodies Used in Immunohistochemical Investigations

Antigen	Clonality	Clone	Supplier
Ki-S1	Mono		Gift Prof H.Kreipe
pS2	Mono		Novocastra
c-*erb*B-2	Mono	CB-11	Novocastra
P- Glycoprotein	Poly		Novocastra
EGFR	Mono	EGFR-1	ICRF
Bcl-2	Mono	124	Dako
p53	Mono	DO-7	Dako

9.15 Oestrogen Receptor Assays

Oestrogen receptor levels were measured by two different assays as a routine clinical service under the direction of Dr.R.A. Hawkins of the Depatment of Surgery, Royal Infirmary of Edinburgh. ERICAs (Oestrogen Receptor Immunocytochemical Assays) were performed on material obtained from diagnostic fine needle aspirates taken prior to treatment. EIAs (Enzyme Immunoassay) were performed on tissue cytosols of tumour specimens obtained from wedge biopsies carried out prior to treatment and, where possible, from specimens obtained at definitive surgery after primary treatment.

9.16 ZR 75.1 Cell Line Xenografts and Treatment with Tamoxifen

The ZR 75.1 human breast cancer cell line, grown as a xenograft was used to monitor the cellular effects of tamoxifen treatment *in vivo* . Established xenograft material was passaged into 18, 8-12 week old, female nude athymic mice (HsdOla:ICRF-nu, purchased from Harlan (UK), Bicester , Oxon) by implanting 1mm tumour fragments subcutaneously into each flank. A pellet of a slow release preparation of oestradiol (0.72mg, released over 60 days supplied by Innovative Research of America, Ohio, U.S.A.) was implanted simultaneously. The tumours were allowed to grow for three to four weeks until at least 5mm in diameter. The mice were divided into three groups and two mice from each group had tumours removed from one flank under anaesthesia.This tissue was then frozen and stored in liquid nitrogen. Fine needle aspirates were taken from a single tumour of each of the remaining mice, the site being carefully recorded. Tamoxifen pellets (drug released over 60 days and supplied by Innovative Research of America, Ohio, U.S.A.) were then implanted subcutaneously in mice belonging to two groups, one group receiving a 5mg pellet, the other 2.5mg. The third group acted as a control. All tumours were measured in two diameters every seven days and fine needle aspirates were performed, also at weekly intervals, on those tumours aspirated prior to treatment. The material obtained by fine needle aspiration was stored in liquid nitrogen and later analysed by flow cytometry for DNA content and the expression of Ki-S1, c-*erb*B-2 and pS2 as described above. Measurements and aspirates were taken at twice-weekly and weekly intervals respectively over a four week period and thereafter the animals were sacrificed and the tumours removed for storage in liquid nitrogen. Tumour material was then processed as described above for immunohistochemistry. Staining was performed with antibodies against Ki-S1, pS2, c-*erb*B-2, *P* - glycoprotein and EGFR.

9.17 T1068 Xenografts and Treatment with Adriamycin

The primary breast cancer xenograft, T1068, established by Dr. F.Balkwill, was used to assess the cellular effects of systemic treatment with adriamycin and their detection by serial fine needle aspiration. Tumour material was passaged by subcutaneous implantation into the flanks of 18 nude mice.The same procedure as described above for hormonally manipulated xenografts was employed although no oestrogen pellets were needed as the T1068 tumour is hormone-independent. The mice were given adriamycin by intraperitoneal injection at doses of 1 and 2mg/kg in the two treatment groups at weekly intervals and control mice given injections of isotonic saline. Fine needle aspirates were taken immediately prior to injection and incubated in BrdU, as described above.The aspirates were stored and later analysed by flow cytometry for DNA content and BrdU uptake. Tumours were measured serially as described above, and all mice were sacrificed after four weeks and the tumour material removed and stored in liquid nitrogen.

RESULTS

Chapter 10 : Cell Line Experiments

Preliminary flow cytometric studies were performed using a variety of established cell lines to optimise and validate methodology. Secondary objectives were to examine the relationship between the different parameters assayed and select those likely to be informative when applied to material derived from fine needle aspirates of primary tumours.

10.1 Markers of Proliferation

As discussed previously in the introductory section there are several different potential methods of assessing cells in the process of division. These include estimation of the nuclear DNA content, the rate of nucleotide (or analogue) incorporation and the levels of antigens expressed specifically during the proliferative phase of the cell cycle by the use of monoclonal antibodies. In the present investigations the potential markers of proliferation that were examined were

a) DNA content and S-phase estimation
b) Incorporation of the thymidine analogue, Bromodeoxyuridine
c) Expression of the nuclear antigens Ki-67 and
d) Ki-S1

These have been studied in two sets of experiments with established cell lines growing i) in culture and described below and ii) in immunocompromised animals as reported in chapter 11. The techniques have then been applied to material obtained by fine needle aspiration of human primary breast tumours (chapter 12)

Cell lines in culture

A series of cell lines of both benign and malignant derivations and different origins were studied. They were selected on account of being available, at the time of the study, in the ICRF Unit of Medical Oncology and are listed in Table 10.1. Cells were grown in standard plastic tissue culture flasks and harvested by trypsin at various phases of growth. The growth phase at harvesting was not controlled as the primary objectives of the experiment were to validate methodologies and compare absolute values for individual proliferative markers at the same time point for each cell line.

Table 10.1 Cell Lines Used in Experiments Examining Markers of Proliferation

Cell Line	Tumour Type	Derivation
ZR 75.1	Breast	Ascites
MCF-7	Breast	Pleural effusion
MCF-7 LS	Breast	LHRH sensitive MCF-7
MDA MB231	Breast	Pleural effusion
HBL 100	Breast	Milk
PEO 1	Ovary	Ascites
PEO 1R	Ovary	Ascites
PEO 4	Ovary	Ascites
PEO 6	Ovary	Ascites
PEO 14	Ovary	Ascites
PEO 23	Ovary	Ascites
OVCAR 3	Ovary	Ascites
OVCAR 4	Ovary	Ascites
OVCAR 5	Ovary	Ascites
2780	Ovary	
2780 AD	Ovary	
2780 PHX	Ovary	
HT 29	Colon	Primary colonic carcinoma
NX002	Lung	Squamous carcinoma xenograft
CX 140	Lung	Squamous carcinoma xenograft
CX 143	Lung	Squamous carcinoma xenograft
Burgen	Fibroblast	Subcutaneous breast fat
Munro	Fibroblast	Subcutaneous breast fat
3T3	Mouse Fibroblast	Swiss mouse embryo
CHO-KI	Hamster Ovary	Adult Chinese Hamster
ADR-R	Hamster Ovary	Exposure to ethyl methane sulfonate
ADR-1	Hamster Ovary	Exposure to ethyl methane sulfonate
ADR-3	Hamster Ovary	Exposure to ethyl methane sulfonate
ADR-6	Hamster Ovary	Exposure to ethyl methane sulfonate

a) S-phase : This was determined after staining of DNA with propidium iodide as described in Section 9.6. Figure 10.1 shows a typical DNA histogram obtained with cells of the ZR 75.1 human breast cancer cell line. The histogram demonstrates two peaks representing nuclei with single and double compliments of DNA. The peaks represent accumulations of cells at the G_0/G_1 phase and the G_2/M phases respectively. Between the peaks lie the cells accumulating DNA in preparation for mitosis, the S-phase, and a computer generated estimate of this area is shown. A total of 29 different cell lines were studied which displayed a wide range of values for S-phase fraction as tabulated in Table 10.2 and shown in Figure 10.2.

Figure 10.1 DNA Histogram Produced by Cells of the ZR75.1 Human Breast Cancer Cell Line Growing in Log Phase

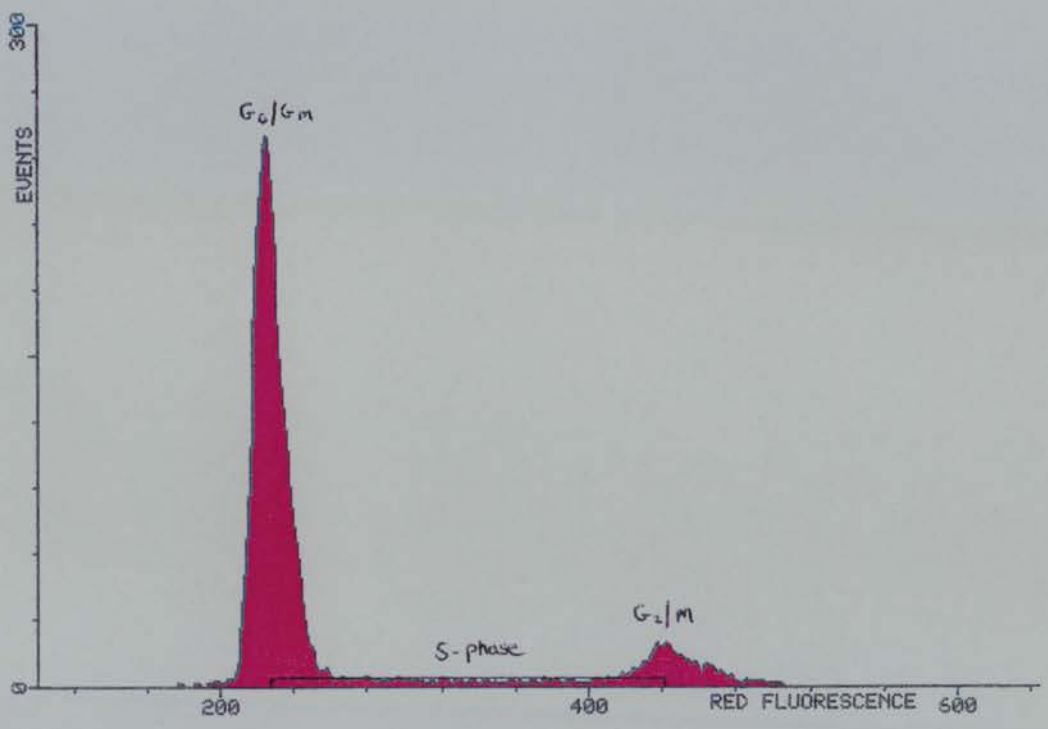


Figure 10.1 shows a typical DNA histogram generated in this instance by propidium iodide staining of the hormone-dependent ZR75.1 human breast cancer cell line. The cells were grown in an oestrogen-supplemented medium and harvested in log-phase.The axes display the number of events (ie. cells) plotted against the quantity of red fluorescence recorded. The different phases of the cell cycle are labelled and a rectangle is superimposed over the S-phase portion of the histogram. This has been generated by the RFIT (**R**ectangular fit) model of the Becton Dickinson cellFIT software and allows an estimation to be made of the small proportion of cells in the G_0/G_1 and G_2/M peaks that are actually in early or late S-phase respectively.

Table 10.2 S-phase and Bromodeoxyuridine Incorporation as Markers of Proliferation in 29 Different Cell Lines

Cell Line	S-phase (%)	BrdU L.I.(%)
ZR 75.1	13.3	13.2
MCF-7	22.0	28.8
MCF-7 LS	33.4	39.8
MDA MB231	26.1	26.6
PEO 1	21.6	29.8
PEO 1R	32.7	21.8
PEO 4	16.9	19.7
PEO 6	13.2	15.3
PEO 14	0.6	0.0
PEO 23	8.0	5.7
OVCAR 3	17.3	18.2
OVCAR 4	33.6	30.0
OVCAR 5	17.5	23.6
2780	22.8	31.5
2780 AD	26.1	32.4
2780 PHX	26.9	34.9
CHO-KI	40.4	36.9
ADR-1	32.3	44.1
ADR-3	52.1	59.5
ADR-6	24.3	13.8
ADR-R	30.0	40.5
NX002	27.2	29.5
HT 29	23.0	29.4
HBL 100	36.1	48.9
CX 140	30.7	28.7
CX 143	34.8	27.9
3T3	5.4	8.9
Burgen	0.8	0.7
Munro	0.2	1.1

Table 10.2 shows values obtained for S-phase fraction (%) and BrdU labelling index (%) in 29 different cell lines. Cells were harvested at random growth phases, incorporated with BrdU and dual stained with propidium iodide and anti-BrdU monoclonal antibody before analysis by flow cytometry.

Figure 10.2 Range of Values for S-phase Fraction Obtained from 29 Different Cell Lines

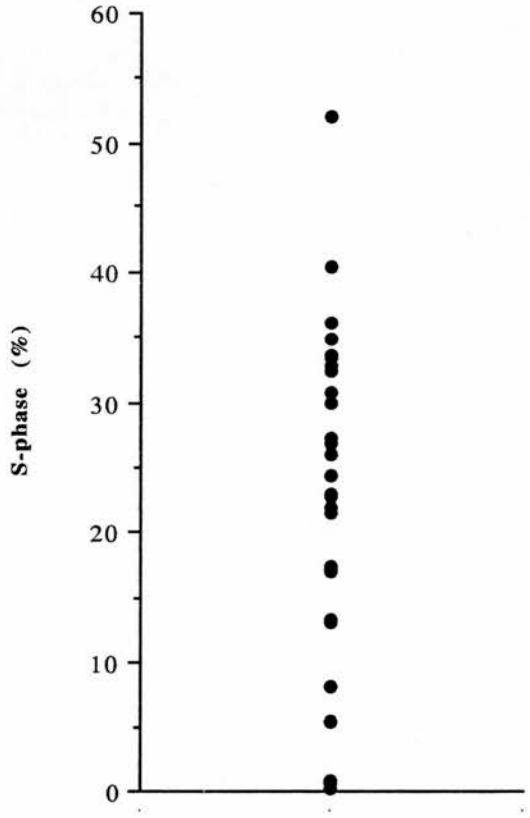


Figure 10.2 shows the range of values obtained for S-phase fraction obtained from DNA histograms generated from the flow cytometer after staining 29 different cell lines with propidium iodide. Each of the cell samples had been dual stained with anti-BrdU antibody after prior incorporation with BrdU.

b) BrdU incorporation : Using the method described in Section 9.7 it was possible to determine a simultaneous estimation of BrdU incorporation and S-phase fraction. BrdU is incorporated into newly synthesised DNA and can be detected by fluorescently labelled monoclonal antibody and flow cytometry. A computer generated dot-histogram (Fig. 10.3), from the analysis of ZR 75.1 cells, shows the typical horseshoe pattern demonstrating the greatest uptake of BrdU (intensity of green fluorescence) at the midpoint of S-phase. Each of the 29 cell lines for which S-phase data has been presented above were also assessed for BrdU incorporation and the absolute values for each parameter are shown in Table 10.2. The relationship between the two indices of proliferation is depicted graphically in Figure 10.4, with there being a highly significant correlation ($p < 0.0001$). Some of these cell lines were also assessed for either Ki-67 or Ki-S1 expression to obtain a comparison of BrdU incorporation and these other indices of proliferation and the results are discussed below.

Figure 10.3 Dot Plots Generated by the Flow Cytometer Demonstrating BrdU Uptake in Relation to the Phase of the Cell Cycle.

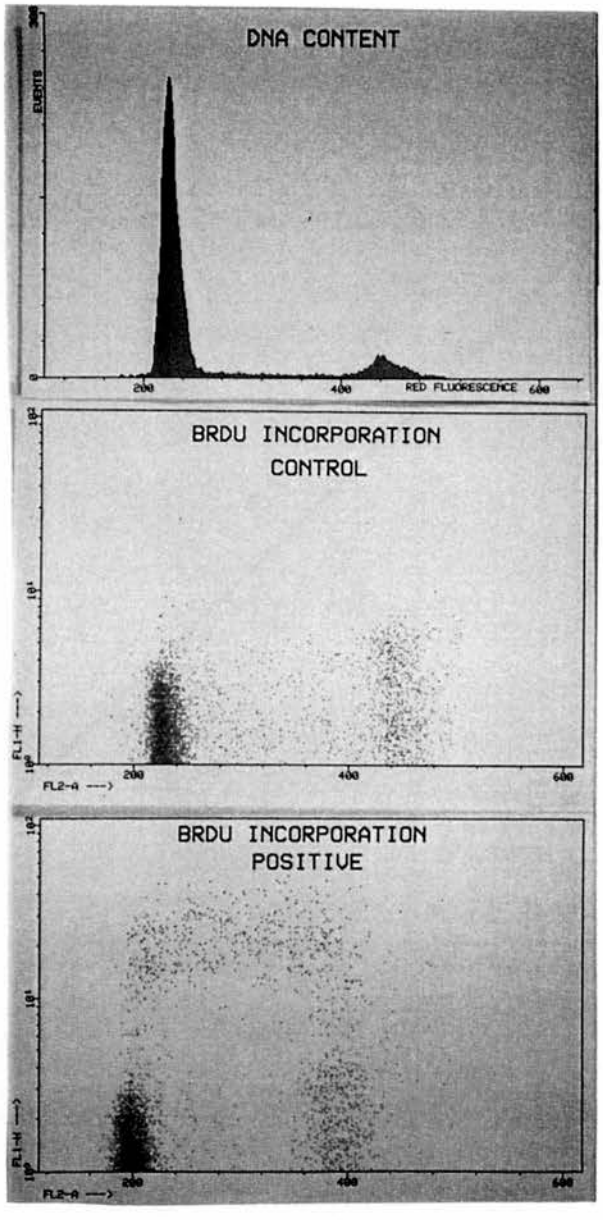


Figure 10.3 shows dot plots of the quantity of green flourescence against red fluorescence in ZR 75.1 human breast cancer cells incubated previously with BrdU. The cells have been stained with a) propidium iodide only as a control and b) with both propidium iodide and fluorescein-conjugated anti-BrdU monoclonal antibody. The increase in green fluoresence over background levels can be seen to peak in those cells whose DNA content (red flourescence) places them in mid S-phase. The DNA histogram, c) is included for reference.

Figure 10.4 The Relationship Between S-phase Fraction and BrdU Labelling Index in 29 Cell Lines.

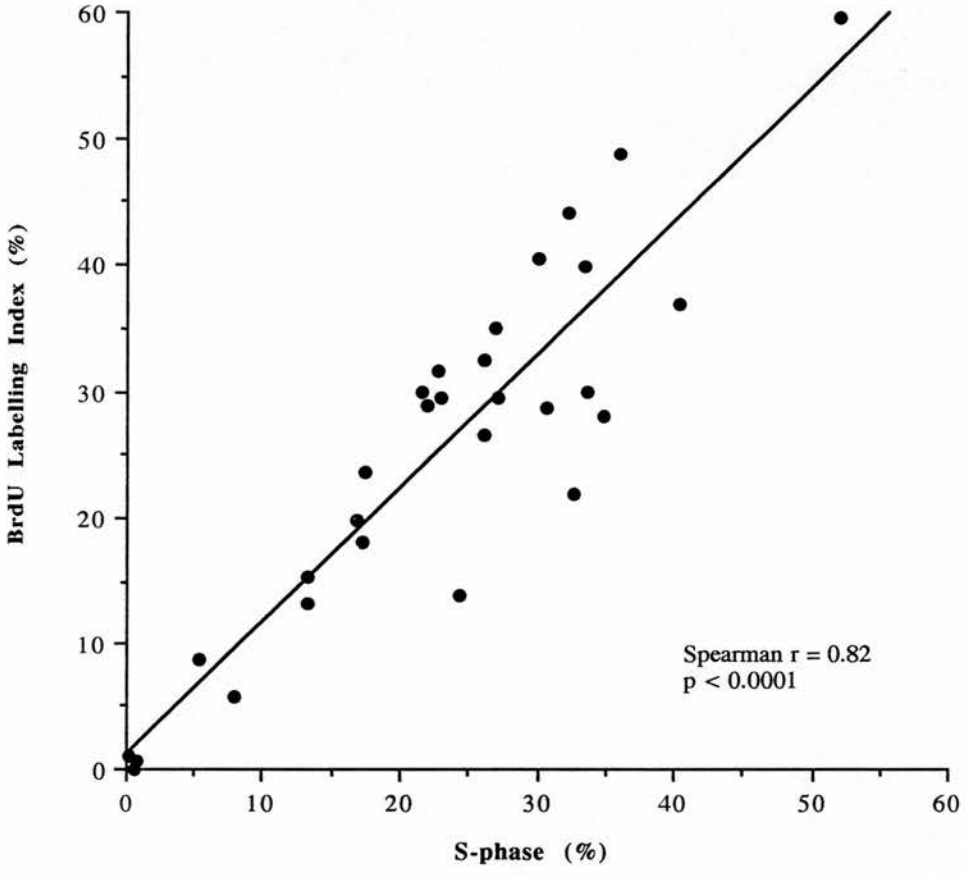


Figure 10.4 shows the relationship between flow cytometric S-phase fraction (expressed as the percentage of cells in S-phase of the cell cycle as computed by the RFIT model of the cellFIT software) and BrdU labelling index (expressed as the percentage of cells showing green flourescence above background in samples incubated with BrdU and stained with FITC-conjugated anti-BrdU monoclonal antibody).

c) Ki-67 expression : Employing the method described in Section 9.8 it was posible to detect this antigen in cell lines and a typical histogram generated by the flow cytometer would be identical to that shown in Figure 10.9 in relation to Ki-S1 expression.

Fourteen of the cell lines described in b) above were also assessed for Ki-67 as a comparison of the methods of determining the rate of proliferation. Cell samples, after incorporation with BrdU to ensure identical time points, were split and either dual stained for BrdU and DNA or for Ki-67 expression. The results are shown in Table 10.3, diagramatically in Figure 10.5, and the relationships between the parameters graphically depicted in Figures 10.6-8. The correlation between Ki-67 and S-phase although significant ($p = 0.013$) was as not as good as that between BrdU incorporation and S-phase ($p = 0.001$). Indeed, Figures 10.5 and 10.6 also suggest that the cell lines can be divided into two groups with "high" and "low" levels of Ki-67 expression and within these two groups the correlation with S-phase is poor. Furthermore the absolute values for Ki-67 labelling index were higher than those for S-phase, probably reflecting the long half-life of this protein, and points to a rôle in determining general proliferative state (or number of "cycling cells") rather than a value for the number of cells accumulating new DNA at a particular point in time. Interestingly the correlation between Ki-67 expression and BrdU incorporation ($p = 0.0005$) was better than that with S-phase (see Figure 10.7) and also, in this particular subset of cell lines, better than the correlation between S-phase and BrdU (Figure 10.8). This may illustrate one of the problems with S-phase in that cells that have arrested with greater than normal amounts of DNA and are no longer cycling or viable at a particular point in time are included in the estimate of S-phase fraction. In theory, therefore, both BrdU incorporation and Ki-67 expression are possibly a more dynamic estimation of the cycling population.

Table 10.3 Cellular Proliferation as Assessed by S-phase, BrdU Incorporation and Ki-67 Expression in Cell Lines

Cell Line	S-phase (%)	BrdU L.I.(%)	Ki-67 L.I.(%)
MCF-7	22.0	28.8	58.8
MCF-7 LS	33.4	39.8	88.4
MDA MB231	26.1	26.6	72.6
PEO 1	21.6	29.8	81.4
PEO 6	13.2	15.3	7.3
PEO 14	0.6	0.0	3.9
PEO 23	5.7	8.0	15.9
NX002	27.2	29.5	59.2
HT 29	23.0	29.4	69.5
HBL 100	36.1	48.9	67.4
CX 140	30.7	28.7	61.9
CX 143	34.8	27.9	38.3
Burgen	0.8	0.7	30.5
Munro	0.2	1.1	9.5

Table 10.3 shows values obtained from 14 different cell lines assessed by flow cytometry for markers of proliferation. Each of the 14 lines were incorporated with BrdU and dual stained with propidium iodide to assess DNA content and particularly S-phase fraction and with monoclonal antibody to BrdU to obtain a labelling index (ie.the number of cells incorporating BrdU and staining with the specific antibody after a 20 minute exposure).The cell lines were also stained with a monoclonal antibody to the Ki-67 antigen and the number of cells staining with a greater fluorescence intensity than background recorded as the labelling index.

BrdU L.I. = Bromodeoxyuridine Labelling Index, Ki-67 L.I. = Ki-67 Labelling Index.

Figure 10.5 The Range of Values Obtained for Ki-67 Labelling Index in 14 Cell Lines

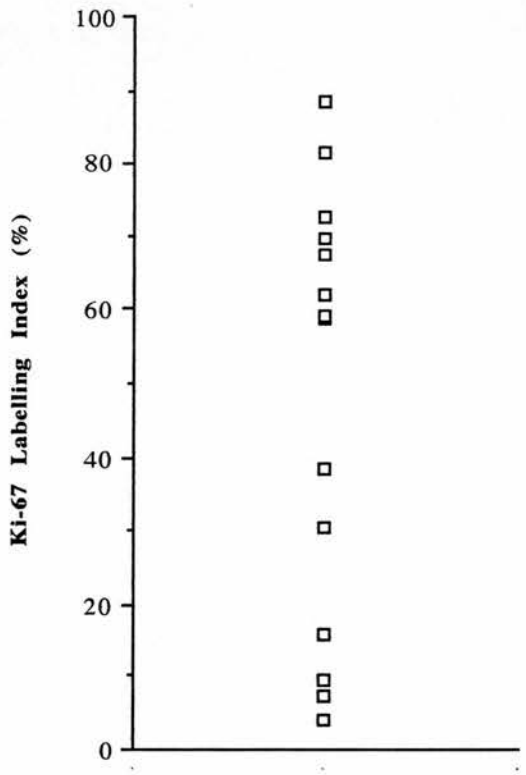


Figure 10.5 shows the range of values obtained of the number of cells staining with a fluorescence intensity over the background level for 14 different cell lines stained with Ki-67 monoclonal antibody.

Figure 10.6 The Relationship Between S-phase Fraction and Ki-67 Labelling Index in 14 Human Cancer Cell Lines

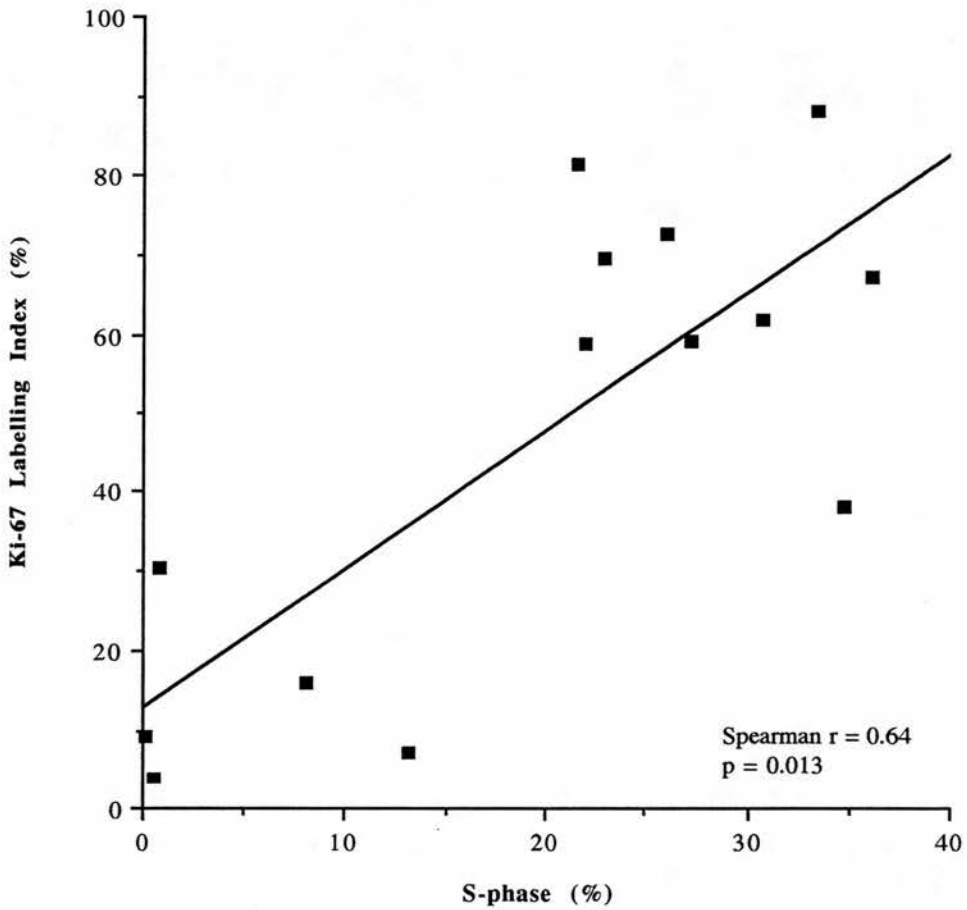


Figure 10.6 shows the relationship between flow cytometric S-phase fraction and the Ki-67 labelling index (expressed as the percentage of cells showing green fluorescence above background control levels after staining with a mouse anti-human Ki-67 monoclonal antibody and visualising with a rabbit-anti-mouse FITC conjugate).

Figure 10.7 The Relationship Between BrdU and Ki-67 Labelling Indeses in 14 Human Cancer Cell Lines

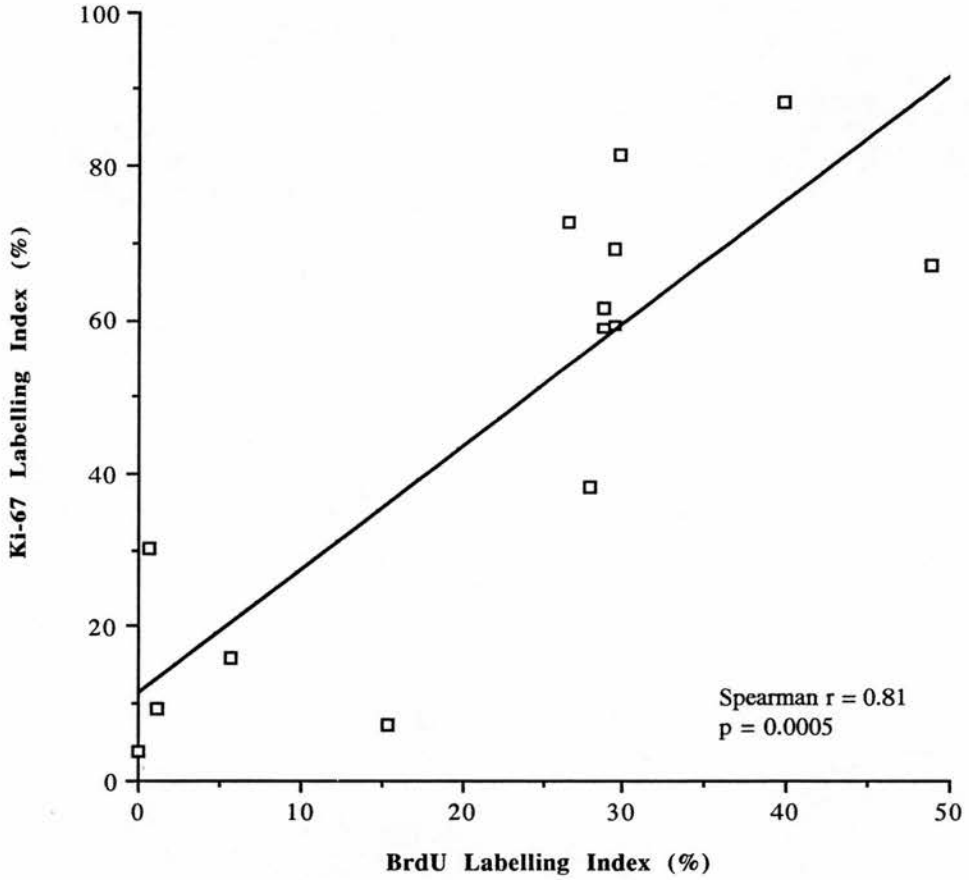


Figure 10.7 shows the relationship between BrdU and Ki-67 labelling indices of a variety of human cancer cell lines. Both markers were measured on cells harvested from the same tissue culture flask at the same time point.

Figure 10.8 The Relationship Between BrdU Uptake and S-phase fraction in a Subset of 14 Human Cancer Cell Lines

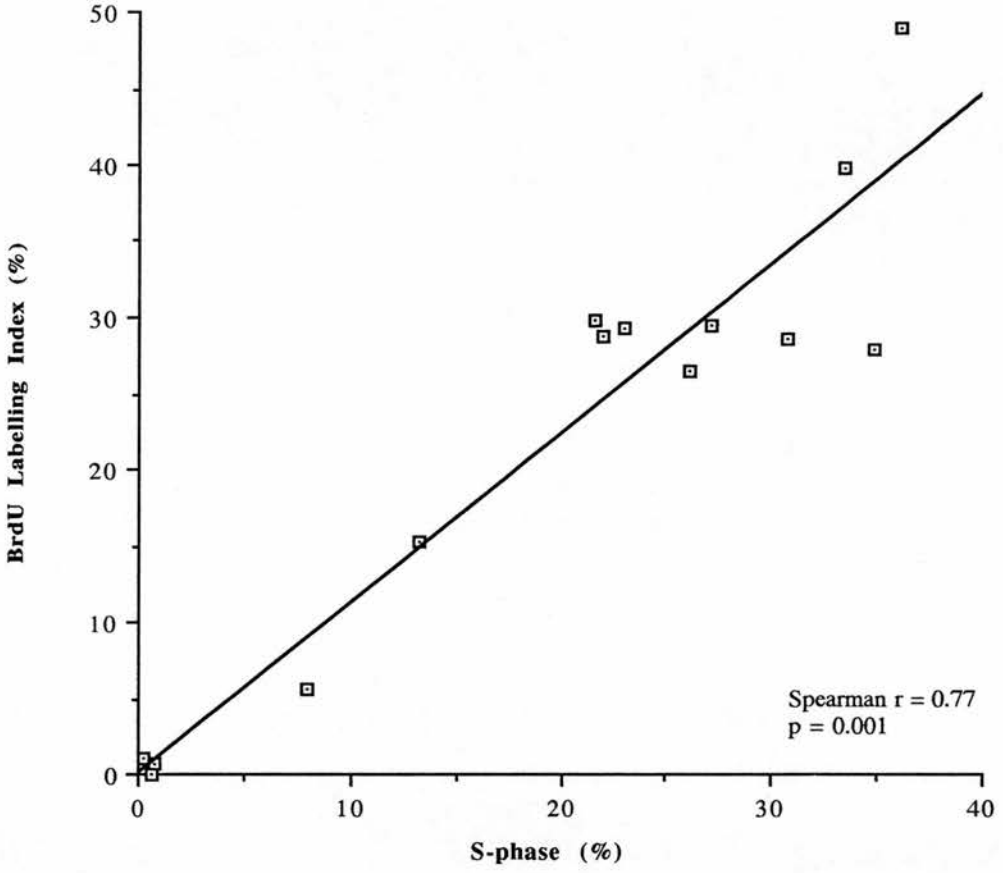


Figure 10.8 shows the relationship between BrdU uptake and S-phase fraction in the subset of cell lines also examined for Ki-67 expression. A statistically significant relationship is noted betwen these two parameters. The correlation is better than that between Ki-67 and S-phase although is not statistically as good as that between BrdU uptake and Ki-67 in this particular subset of 14 cell lines. This may demonstrate the different approach to the assessment of proliferation that each of these markers represents.

d) Ki-S1 : This antibody, in a similar fashion to Ki-67, reported above, detects a nuclear antigen expressed during the proliferative phases of the cell cycle. Ki-67 produced poor results with clinical material that had been frozen during storage (see chapter 12), a finding confirmed by others (R. Camplejohn, Personal Communication). The antigen for the relatively new antibody, Ki-S1 had been shown to be much more stable and could also be stained by immunohistochemistry in paraffin-embedded tissue sections, another advantage over Ki-67.

To assess this new antibody a similar study design to those described previously was adopted in which Ki-S1 expression was compared to S-phase fraction and BrdU incorporation in a series of cell lines. A random group of 18 of the cell lines previously described were incorporated with BrdU and then two aliquots were either dual stained with anti-BrdU antibody and propidium iodide or stained singly with Ki-S1 antibody. A typical histogram generated by the flow cytometer is shown in Figure 10.9. The results of the experiment are shown in Table 10.4 and diagramatically displayed in Figure 10.10.The results are similar to those obtained for Ki-67 antibody, although the correlation between Ki-S1 and S-phase was not statistically significant there were significant correlations between BrdU incorporation and S-phase ($p = 0.0004$) and Ki-S1 and BrdU incorporation ($p = 0.03$) as shown in Figure 10.11.

Figure 10.9 Ki-S1 Expression in the ZR-75.1 Breast Cancer Cell Line

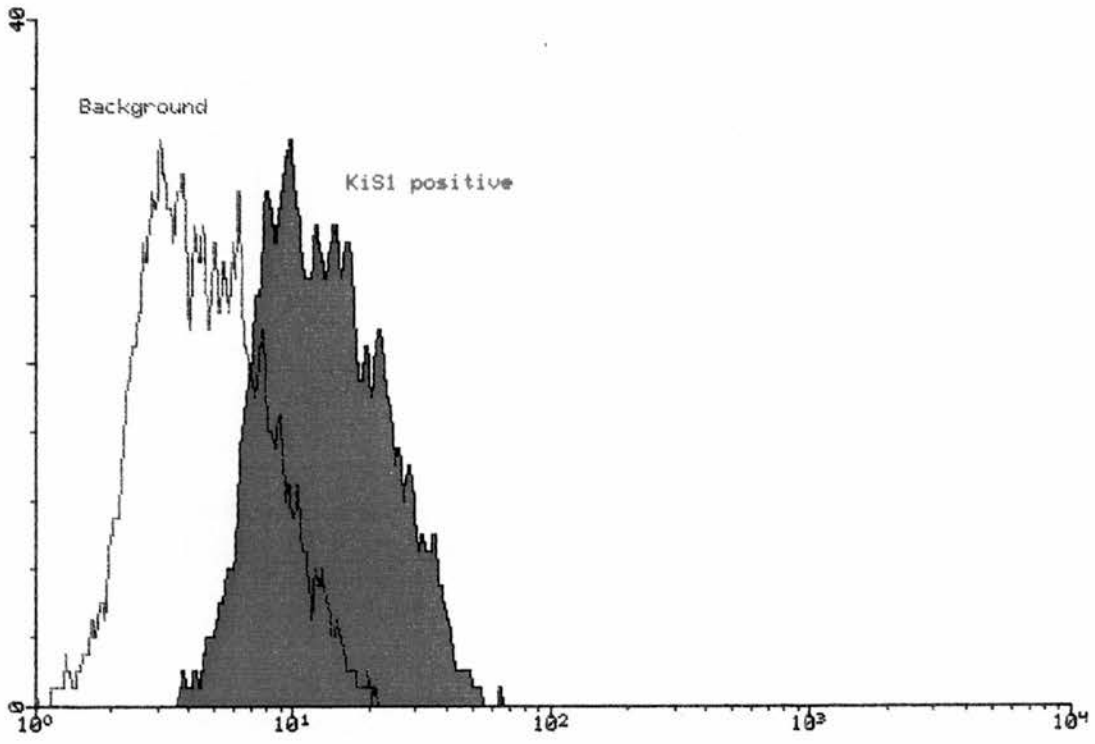


Figure 10.9 shows superimposed histograms generated by the flow cytometer. The number of events (cells) counted is plotted against the intensity of green fluorescence. The mean fluorescence is seen to be greater in the sample of cells stained with Ki-S1 antibody (and exposed to a fluorescein label) than in control cells. The control cells demonstrate background fluorescence and the percentage of cells in the test sample with fluorecence greater than background levels is termed the labelling index.

Table 10.4 The Expression of Ki-S1 and the Relationship With Other Proliferative Markers in Cell Lines

Cell Line	S-phase (%)	Ki-S1 L.I.(%)	BrdU L.I.(%)
ZR 75.1	5.7	18.6	3.5
MDA MB231	10.1	40.8	10.2
PEO 1	21.8	20.2	20.1
PEO 1R	32.7	21.5	21.8
PEO 4	17.7	34.5	24.3
OVCAR 3	17.3	0.0	18.2
OVCAR 4	33.4	9.0	30.0
OVCAR 5	17.5	20.3	23.6
2780	22.8	33.3	31.5
2780 AD	26.1	98.0	32.4
2780 PHX	34.9	4.9	26.9
CHO-KI	40.4	19.0	36.9
ADR-1	32.3	41.3	44.1
ADR-3	52.1	56.3	59.5
ADR-6	24.3	0.0	13.8
ADR-R	30.0	74.3	40.5
NX002	26.4	42.5	14.5
3T3	9.4	0.0	13.1

Table 10.4 shows the results obtained from flow cytometric assessment of proliferation in 18 different cell lines.

BrdU L.I. = Bromodeoxyuridine Labelling Index, Ki-S1 L.I. = Ki-S1 Labelling Index.

Figure 10.10 The Range of Values Obtained for the Expression of Ki-S1 in 18 Different Cell Lines

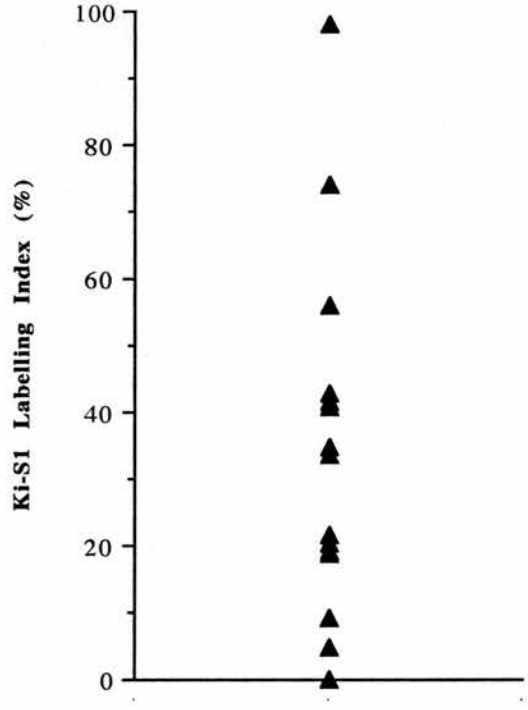
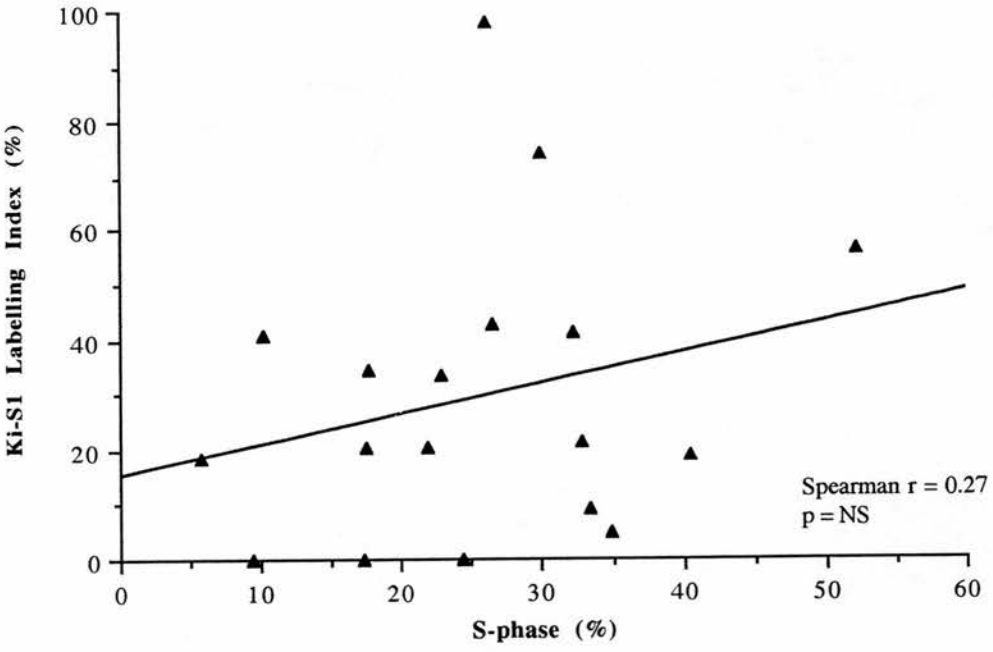
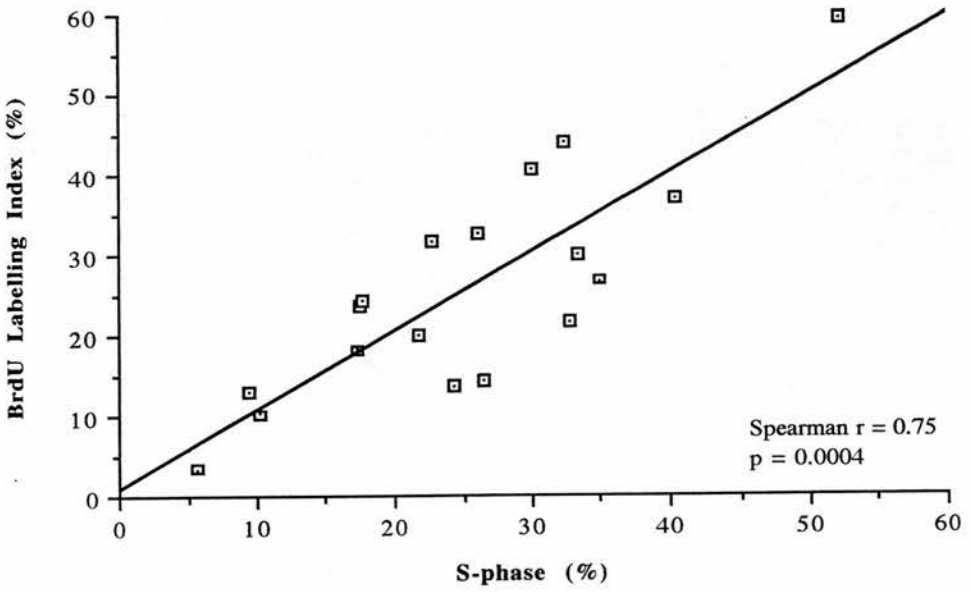


Figure 10.10 shows the ranges of values obtained for the percentage of cells staining with a fluorescent intensity above the control background levels. The test cells having been stained with a fluorescent-labelled Ki-S1 monoclonal antibody.

Figure 10.11 The Relationships Between S-phase, Ki-S1 Labelling Index and BrdU Labelling Index in 18 Different Cell Lines

i)





iii)

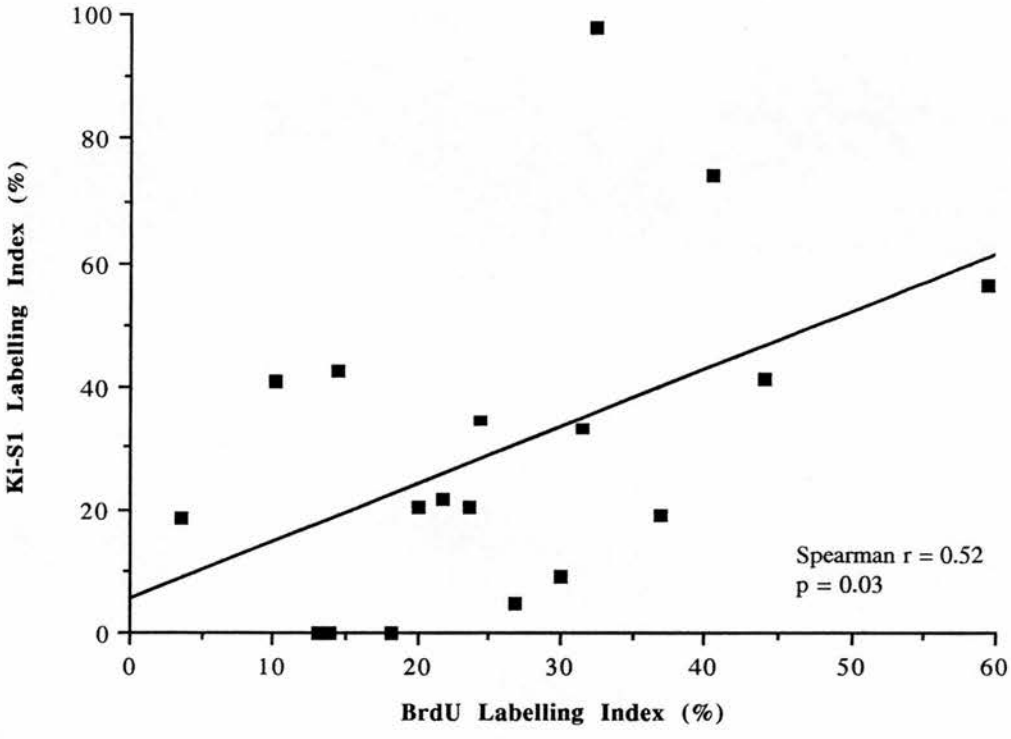


Figure 10.11 shows the relationships between three markers of proliferation measured in 18 different cell lines; **i)** A lack of correlation between Ki-S1 expression and S-phase fraction, **ii)** a highly significant correlation between BrdU incorporation and S-phase and **iii)** a significant correlation between Ki-S1 expression and BrdU incorporation.

Summary

These initial experiments with randomly selected cell lines allowed the development of methodology and the study of the inter-relationships between three very different methods of assessing cellular proliferation by flow cytometry. A consistent finding in this model was of the highly significant correlation between the S-phase fraction as assessed by cellular DNA content and the fraction of cells incorporating BrdU. The expression of the nuclear proliferation-associated antigens, Ki-67 and Ki-S1 showed better correlations with BrdU incorporation than with S-phase fraction.

The estimation of S-phase fraction has until recently been felt to be the most accurate tool of cellular proliferation assessment in flow cytometry. With some debate over this and the results to suggest that there is not a simple relationship between these parameters it was felt to be important to pursue the measurement of S-phase, BrdU incorporation and expression a nuclear proliferation antigen into a clinical setting. Initially Ki-67, and then Ki-S1 expression was measured in clinical material when the latter antibody became available and after problems of Ki-67 stability had become apparent.

In order to assess the suitability of these techniques for measuring proliferation in solid tumours, breast tumour xenografts in immunocompromised mice were studied. This also afforded the opportunty of assessing these measurements as a reflection of response to systemic anti-tumour therapy and the results are reported in the following chapter.

Chapter 11: Xenograft Experiments

This study was performed to test the methodology of assessing cellular proliferation, as developed in tissue culture systems, in a solid tumour model with material obtained by fine needle aspiration. There have been no previous reports, to our knowledge, of the technique of fine needle aspiration being employed to obtain material from animal xenografts and particularly for the assessment of response to systemic therapy.

11.1 ZR 75.1 Xenografts and the Response to Tamoxifen

Nude mice with established bilateral flank subcutaneous ZR 75.1 xenografts were allocated to three groups; a control, untreated group, and two treated groups. The two treated groups were given treatment by the subcutaneous implantation of either a 2.5mg or a 5mg slow release capsule of tamoxifen. Fine needle aspirates were performed on the same tumour from each mouse on day 7, 14, 21 and 28 after the start of the experiment.Tumour volumes were calculated from two diameter measurements taken at weekly intervals and charted (Table 11.2 and Fig 11.2).

All four tumours in the control group showed an increase in volume during the experiment although growth occurred at differing rates and one tumour decreased in size between the penultimate and final time point. Tumours treated with 2.5mg of tamoxifen all showed an increase in size (allbeit smaller than those in the control group) until day 14 and thereafter a reduction in volume. Three of the four tumours treated with 5mg of tamoxifen showed an increase at day 7 and thereafter a decrease in volume. The fourth tumour displaying a decrease in volume at day 7.

There was a greater incidence of ulceration in those tumours treated with the higher dose which made measurement and fine needle aspiration more difficult. Both doses tended to cause a fibrotic reaction with the tumours becoming pale and firm. This may be a reason for the relatively small change in volume noted in treated tumours, but one might also interpret this observation as typical of the reaction to a supposed cytostatic agent.

Serial measurements of S-phase on cellular material obtained by fine needle aspiration showed clear differences between treated xenografts and controls (Table 11.2 and Fig 11.3). The S-phase fractions of control tumours remained constant or increased marginally throughout the experiment wheras those from treated tumours decreased progressively. The S-phase fraction of tumours treated at the higher dose of 5mg of tamoxifen fell, initially, significantly faster than the lower dose. This may reflect the

relative delay in response of the tumours treated at the lower dose as observed by the serial measurements reported above. There was no significant difference, by the end of the experiment, in either tumour volume or S-phase of the two treated groups.

The specimens obtained one week after commencement of treatment were spoiled because of overheating whilst incorporating BrdU, due to a faulty thermostat. Insufficient data was obtained to meaningfully report serial BrdU incorporations. The relatively small size and firm consistency of the ZR 75 xenograft resulted in difficulty in consistently obtaining sufficient material for the multi-step preparation required for anti-BrdU staining.

Table 11.2 Results of the Treatment of ZR 75.1 Xenografts in Nude Mice with Tamoxifen

Mouse	Day	Volume (cm^3)	S-phase (%)
1a	0	0.209	10.3
(Control)	7	0.332	
	14	0.540	8.5
	21	0.878	14.9
	28	1.081	16.5
2a	0	0.449	7.9
	7	0.541	
	14	0.595	5.1
	21	0.653	14.9
	28	0.713	12.5
3a	0	0.433	12.5
	7	0.587	
	14	0.972	13.4
	21	1.039	5.2
	28	0.561	5.0
4a	0	0.367	14.4
	7	0.571	
	14	0.908	13.8
	21	1.104	13.7
	28	1.391	19.6
1b	0	0.342	13.8
(2.5mg	7	0.343	
Tamoxifen)	14	0.421	4.1
	21	0.303	4.4
	28	0.299	6.3
2b†	0	0.117	9.4
	7	0.134	
	14	0.113	2.5
	21	0.059	2.1
	28	0.063	

Table 11.2 Continued

3b	0	0.359	18.1
	7	0.486	
	14	0.513	11.6
	21	0.413	3.4
	28	0.392	2.8
4b*	0	0.455	
	7	0.515	
	14	0.535	17.9
	21	0.428	5.3
	28	0.327	10.7
1c (5mg Tamoxifen)	0	0.340	9.6
	7	0.325	
	14	0.241	2.0
	21	0.221	2.7
	28	0.225	1.5
2c**	0	0.259	12.9
	7	0.470	
	14	0.510	6.0
	21	0.272	8.9
3c	0	0.356	20.3
	7	0.530	
	14	0.460	4.4
	21	0.259	4.3
	28	0.240	4.0
4c†	0	1.048	11.9
	7	1.054	
	14	0.721	5.0
	21	0.444	6.6
	28	0.343	

† Tumour fibrosed at day 28 and unable to obtain adequate FNA.

* Acellular FNA day 0.

**Tumour necrosis necessitating sacrifice of mouse prior to day 28.

Table 11.2 shows the raw data obtained from each of the twelve ZR 75.1 xenografts studied. Group a) acted as controls, group b) treated with a 2.5mg implant of tamoxifen and group c) a 5mg implant. Tumour volumes were calculated from two diameter calliper measurements taken at weekly intervals. Fine needle aspirates were taken weekly and S-phase fractions calculated from flow cytometric DNA histograms obtained after propidium iodide staining. Dual staining enabled a BrdU labelling index to be calculated in those samples generating sufficient cellular material. The data for the individual tumours from each group have been combined and are graphically depicted in figure 11.3.

Figure 11.2 The Response of ZR 75.1 Xenografts in Nude Mice to Treatment with Tamoxifen

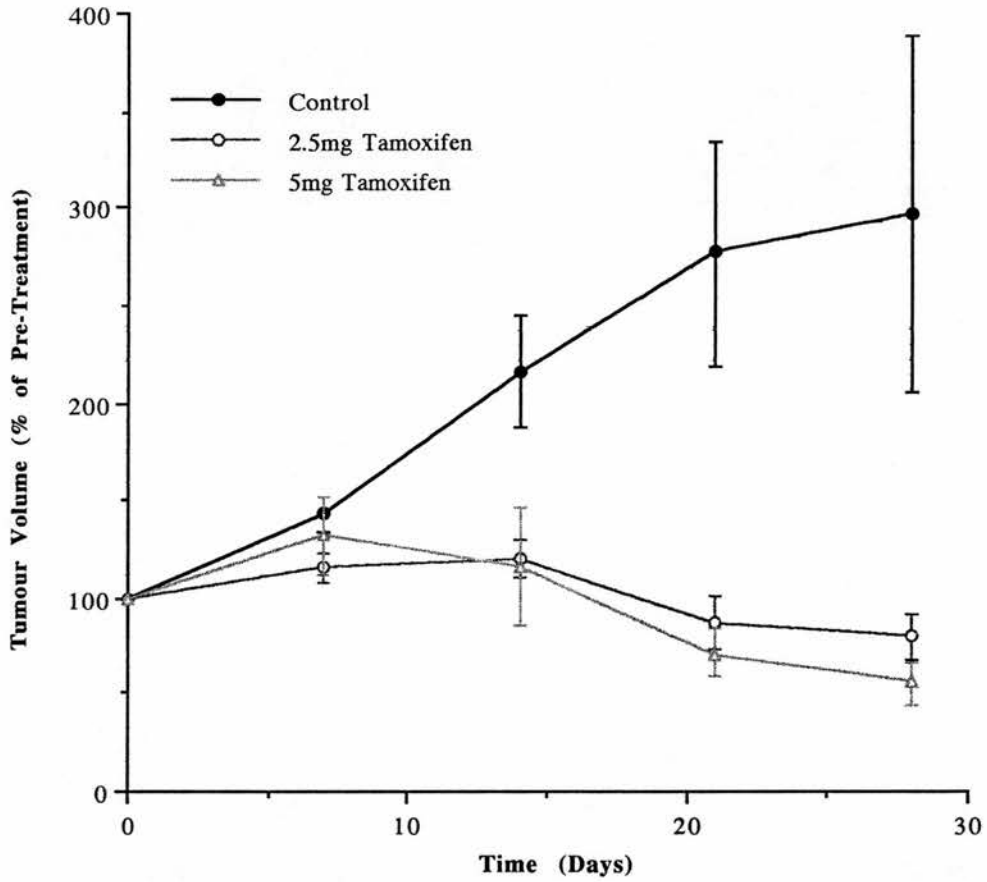


Figure 11.2 shows the change in mean tumour volume, expressed as a percentage of that measured immediately prior to commencement of tamoxifen, of assessable xenografts in four control mice and two other groups of four mice treated with 5mg and 2.5mg of tamoxifen respectively. There is a clear difference between control and treated xenograft; tamoxifen causing an initial decrease in the rate of growth and then a decrease in tumour volume, but there is no significant difference between the two doses of tamoxifen in the response observed.

Figure 11.3 Serial S-phase Measurements in ZR 75.1 Xenografts in Nude Mice Treated with Tamoxifen

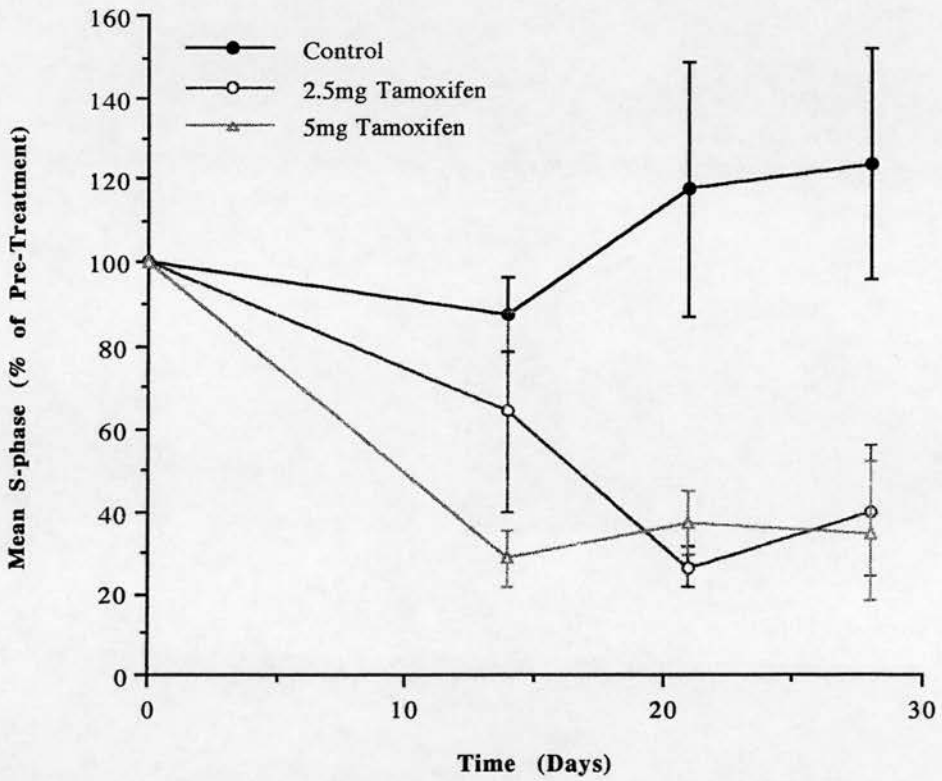


Figure 11.3 shows the change in S-phase fraction as assessed by flow cytometry and DNA analysis of serial fine needle aspirates taken from ZR 75.1 xenografts in groups of four nude mice treated with 5mg, 2.5mg or no tamoxifen. The data from which the plot for the group treated with 2.5mg of tamoxifen was calculated was derived from three xenografts as the pre-treatment specimen from the fourth tumour was unassessable. The S-phase fractions in the control group remained stable wheras those in the treated groups decreased corresponding to the decrease in tumour size (see figure 11.2). The S-phase fraction of xenografts treated with 5mg of tamoxifen appeared to drop significantly faster than those treated with only 2.5mg.

11.2 T1068 Xenografts and the Response to Adriamycin

In an attempt to determine the feasibility of observing the tumour biological response to adriamycin by serial fine needle aspiration a similar experiment to that described above was devised. The human breast cancer xenograft T1068 was used, being derived from a mucoid carcinoma of the breast in an untreated postmenopausal woman (Balkwill, 1984). This xenograft, grows very quickly and forms a soft cellular tumour from which it is easy to obtain relatively good samples by fine needle aspiration (unlike the ZR 75.1 xenografts descibed above) and is sensitive to adriamycin. Eight nude mice with established tumours were divided into two groups. The first group acting as controls were given weekly intra-peritoneal injections of saline and the other were treated by intra-peritoneal adriamycin at a dose of 0.5mg/kg. Tumour volumes were calculated by two-diameter calliper measurements taken weekly and charted (Table 11.4 and Fig. 11.4).

There was no overall significant difference in the growth rate of treated and control mice as shown in Figures 11.4 and 11.6. However mean tumour volumes of the treated tumours at seven and 14 days were significantly lower than those of the control tumours.

Fine needle aspirates of the T1068 xenograft yielded sufficiently cellular material to enable both the BrdU labelling index and S-phase to be measured for the majority of samples (Table 11.4). This allowed a more detailed examination of the relationships between observed response and the two indices of cellular proliferation. Figure 11.5 shows the variation of the mean values for these two parameters over time and Figures 11.6 to 11.8 show graphically the inter relationships for each individual xenograft studied. Interestingly the S-phase fraction declined with time in both the treated and the control tumours (Figures 11.5 and 11.7). The values for S-phase in individual tumours declined progressively in the control tumours, whereas in the treated tumours, whilst the trend is towards a decrease, the values for serial S-phase estimations are more erratic. A similar observation was noted with the serial measurements of BrdU L.I. although the curves for treated tumours are relatively smoother than those described previously for S-phase (Figure 11.8). Interestingly each of the treated tumours showed an increase in BrdU L.I. (but not S-phase) at day 7 compared to the pre-treatment value. This might be a demonstration of the initial stimulation of proliferation noted by some investigators on exposure of cell lines to adriamycin (Dr. J. Cummings, Personal Communication).

The apparent difference in serial S-phase and BrdU L.I. measurements in the treated tumours as opposed to the controls and the inferred poor correlation between S-phase

and BrdU L.I. can be demonstrated by plotting the two indices against each other as shown in Figures 11.9 and 11.10. Figure 11.10 represents combined data from each of the tumours in a) the control and b) the treated group. The data from the control group demonstrates the statistically significant relationship between S-phase and BrdU, previously shown in cell line work above. This significance, however, is lost in the samples from treated tumours.

It became apparent from this work on the measurement of cellular proliferation in xenografts during systemic treatment that at least two approaches would be important in monitoring the clinical response to treatment. Therefore these techniques were applied to specimens obtained from primary breast tumours with the results as described in the following chapter.

Table 11.3 The Response of T1068 Xenografts in Nude Mice Treated with a) Saline as a Control and b) 0.5mg/kg Adriamycin

Mouse	Day	Volume (cm^3)	S-phase (%)	BrdU L.I.(%)
1a	0	0.247	29.1	16.46
(Control)	7	0.622	14.4	11.37
	14	0.989	11.9	6.35
	21	1.512	9.9	4.29
	28	2.745	8.4	2.67
	31	2.807		
	35		7.5	2.42
2a	0	0.297	38.3	12.57
	7	0.657	23.3	15.71
	14	0.922	18.6	4.52
	21	2.065	19.6	3.81
	28	2.094		0.11
	31	1.461		
	35			0.60
3a	0	0.085	41.9	
	7	0.363	33.5	13.76
	14	0.631	29.5	6.79
	21	0.936	17.9	5.09
	28	2.207	21.2	3.74
	31	1.461		
	35		20.1	5.10
4a	0	0.060	37.8	15.90
	7	0.112	25.4	12.24
	14	0.171	18.4	7.79
	21	0.358	15.1	5.00
	28	0.700	16.5	6.29
	31	0.806		
	35		9.7	2.65

Table 11.3 Continued

Mouse	Day	Volume (cm^3)	S-phase (%)	BrdU L.I.(%)
1b	0	0.144	12.6	4.92
(.5mg/kg	7	0.215	14.0	12.45
Adriam'n)	14	0.174	19.4	12.10
	21	0.320	19.5	9.60
	28	0.475	21.0	8.00
	31	0.397		
	35		13.6	11.57
2b	0	0.011	36.9	14.45
	7	0.012	31.2	16.83
	14	0.027	37.9	10.15
	21	0.071	34.6	13.97
	28	0.144	28.8	12.19
	31	0.167		
	35		38.0	11.18
3b	0	0.124	39.3	12.25
	7	0.168	18.6	15.13
	14	0.262	27.9	11.56
	21	0.646	12.7	8.43
	28	0.807	18.5	5.37
	31	0.864		
	35			5.32
4b	0	0.252	40.9	9.97
	7	0.561	32.6	15.41
	14	0.863	31.3	6.71
	21	1.701	23.1	2.01
	28	2.596	11.6	3.15
	31	2.633	11.6	
	35			1.05

Figure 11.4 Growth Curves of the T1068 Human Breast Cancer Xenograft in Nude Mice Treated with Adriamycin (0.5mg/kg)

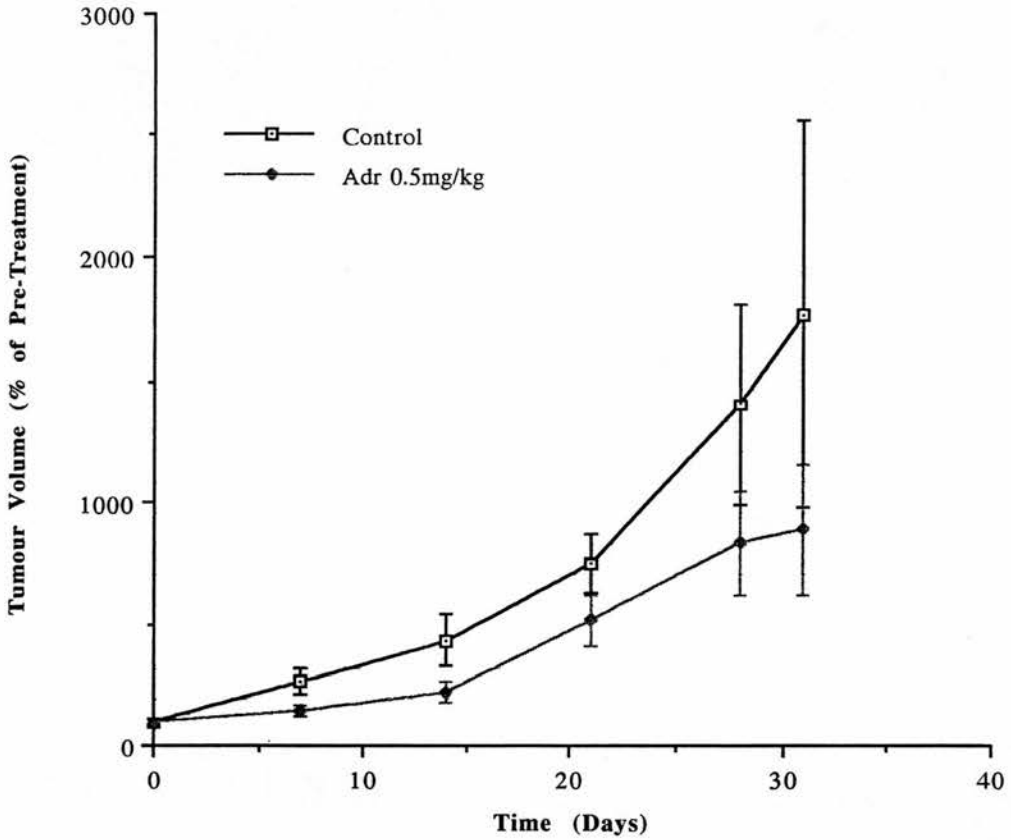
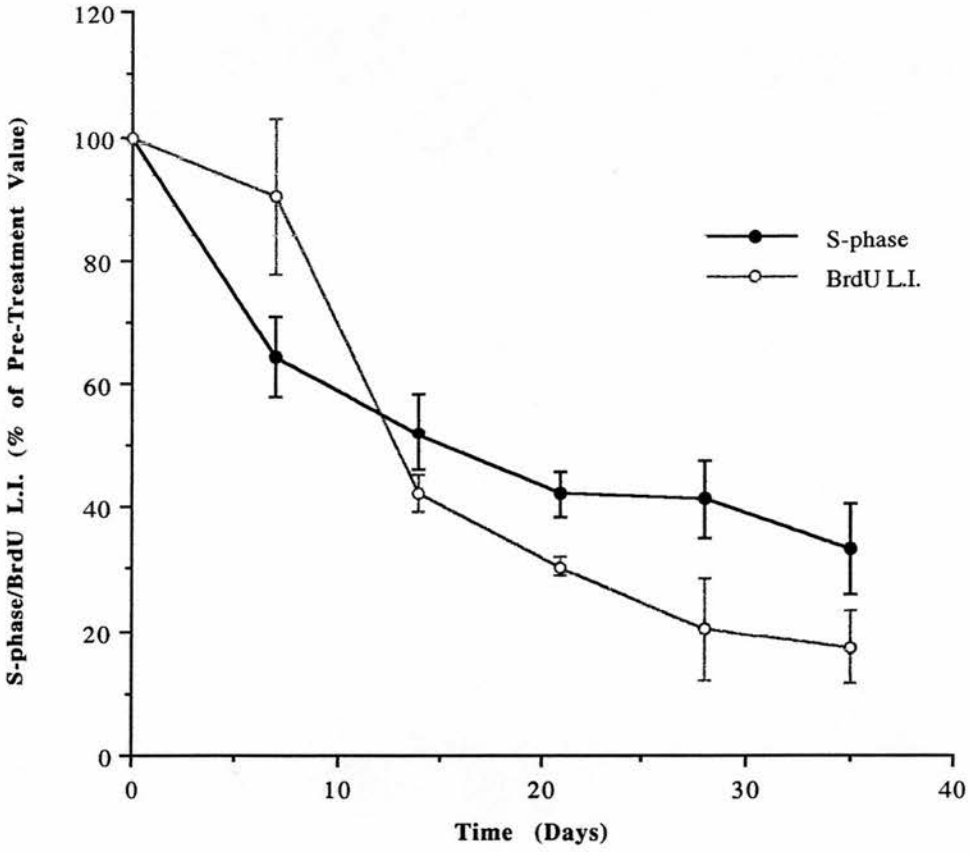


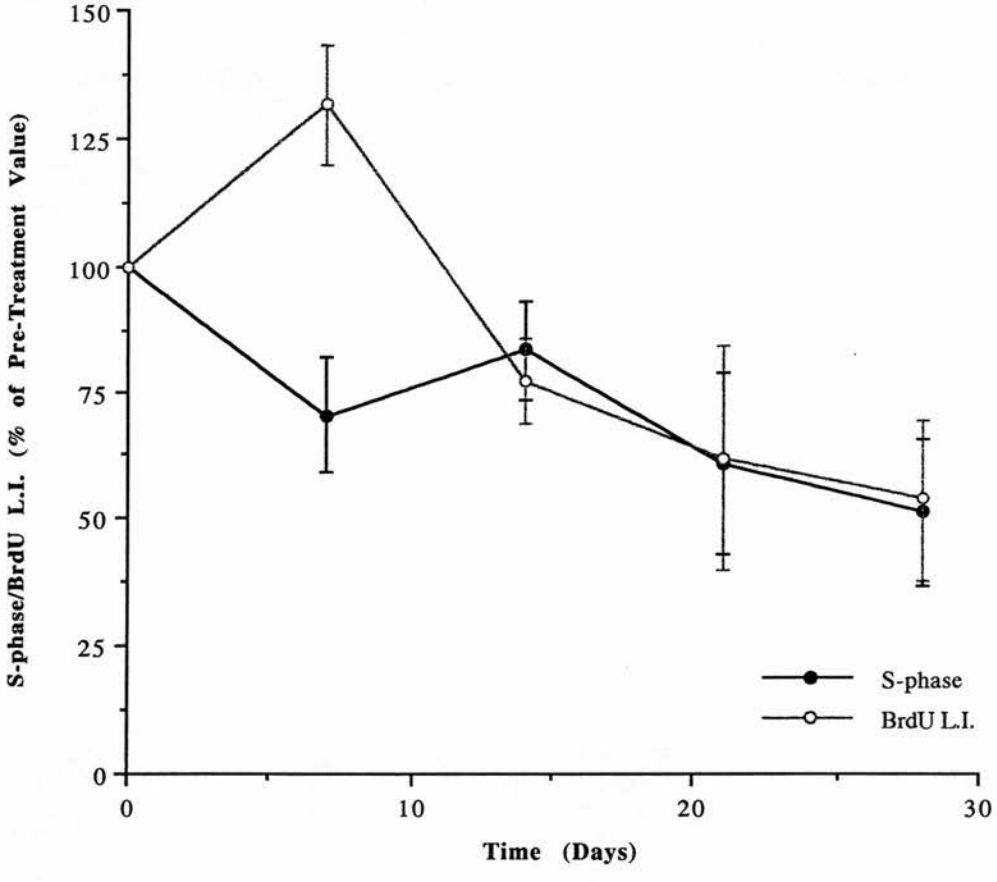
Figure 11.4 shows the growth curves produced by serial tumour mesurement prior to weekly fine needle aspirates. Treated animals were given 0.5mg/kg adriamycin by intra-peritoneal injection and controls were simply given the same volume of 0.9% saline. The results shown are for five mice in each group. Given the broad standard Error bars there appears to be no significant effect of adriamycin on the growth curve at this dose after 31 days of treatment.

Figures 11.5a and 11.5b Serial Measurements of S-phase and BrdU Labelling Index in T1068 Xenografts a) Untreated Controls and in b) Treated with Adriamycin

11.5a



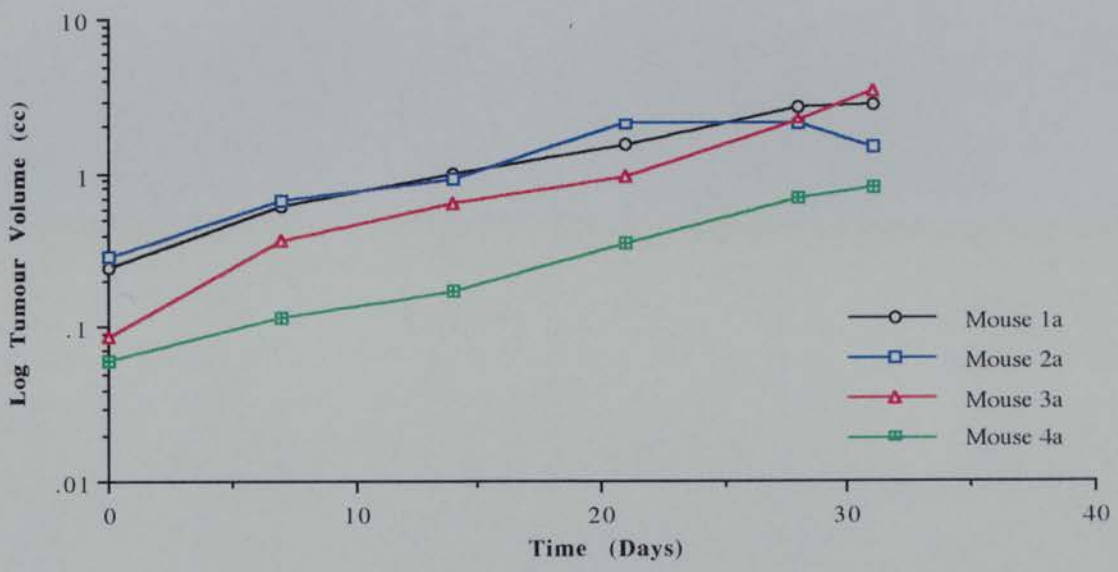
11.5b



Figures 11.5a and 11.5b show the change in the mean values of S-phase fraction and BrdU uptake over time in four xenografts from each of control and treated groups of mice. Both parameters show a decline over time in each group. The only significant difference between the groups appears to be an initial increase in BrdU uptake (but not S-phase) in the treated tumours.

Figure 11.6 Growth Curves of Individual T1068 Xenografts; a) Controls and b) Treated with 0.5mg/kg Adriamycin.

a



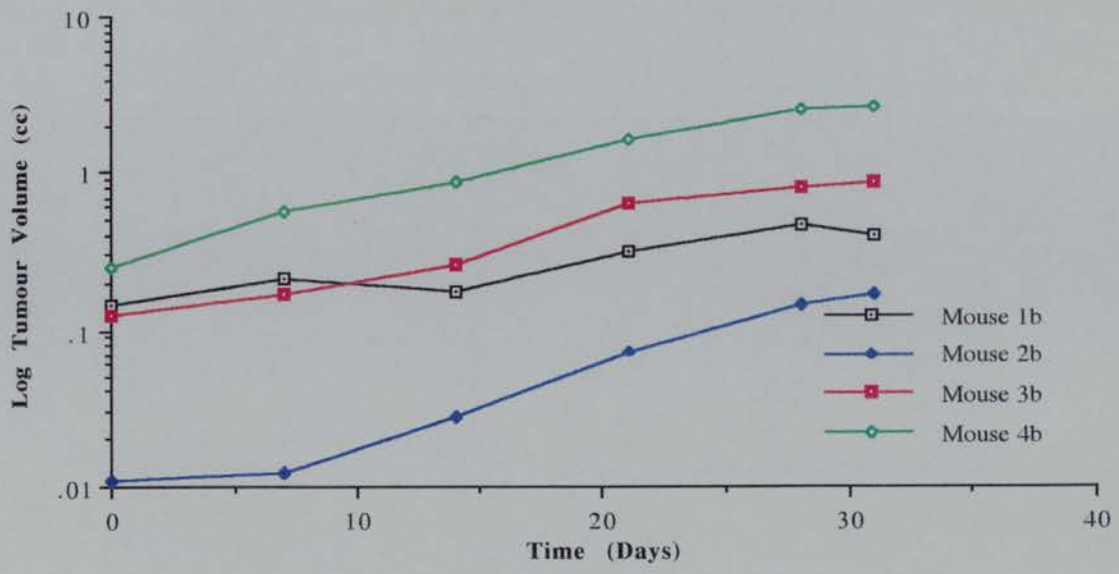


Figure 11.6 shows the growth curves of each of the four xenografts in the control group (a) and those treated with 0.5mg/kg Adriamycin (b). On a log scale each tumour displays linear growth with little difference in growth pattern between controls and those treated with adriamycin.

Figure 11.7 The Relationships between S-Phase Fraction and Time in Individual T1068 Xenografts; a) Controls and b) Treated with 0.5mg/kg Adriamycin

a

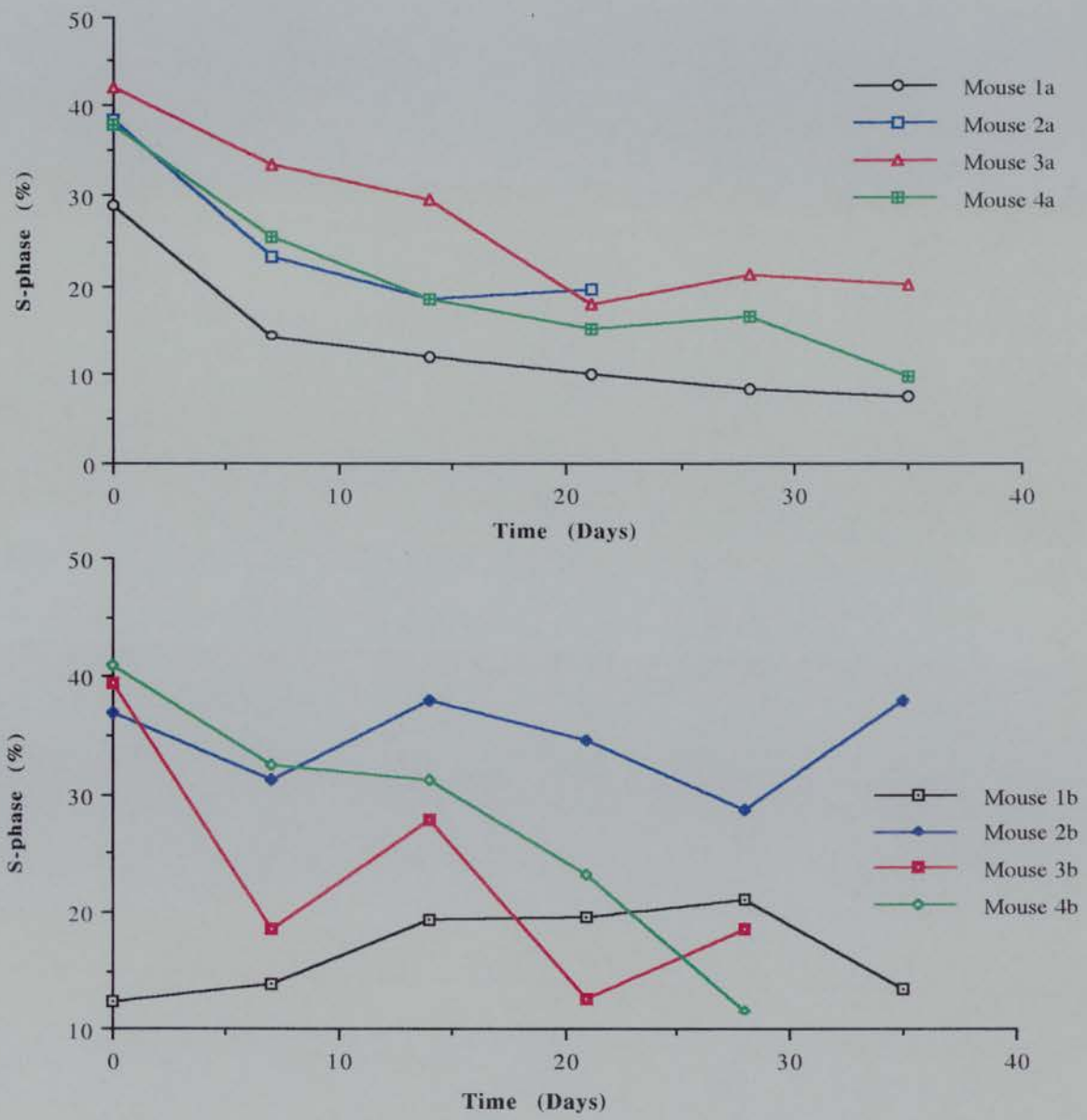
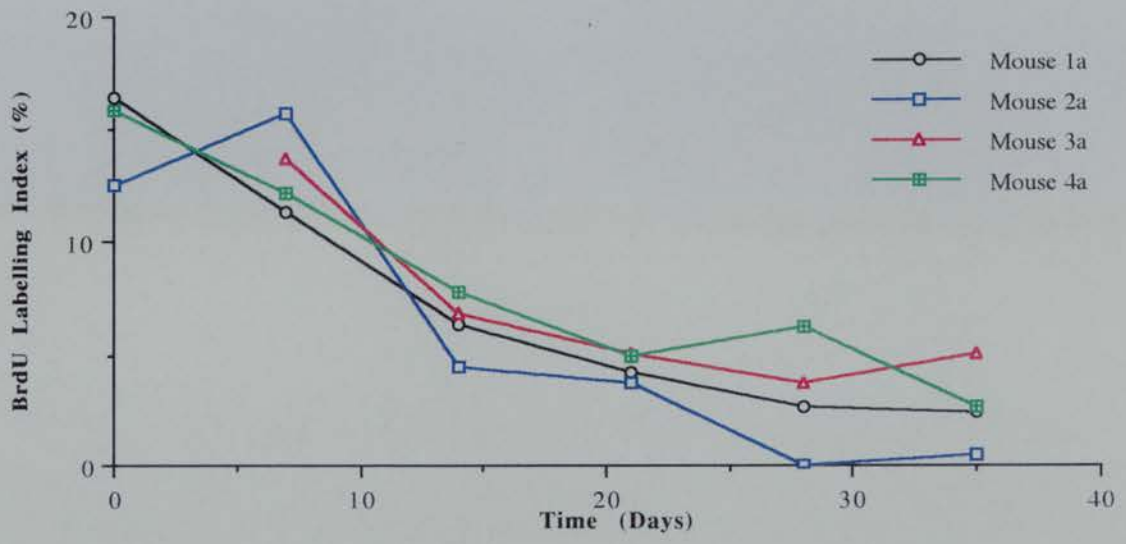


Figure 11.7 shows the variation with time of S-phase fraction as determined from DNA histograms generated by flow cytometry of serial fine needle aspirates taken from a) control tumours and b) tumours treated with 0.5mg/kg Adriamycin. S-phase tends to decrease with time in both the control and treated tumours despite the fact that both groups showed tumour growth over this time period. The variation of S-phase is more uniform in the controls compared to those undergoing treatment .

Figure 11.8 The Relationships between BrdU Labelling Index and Time in Individual T1068 Xenografts; a) Controls and b) Treated with 0.5mg/kg Adriamycin

a



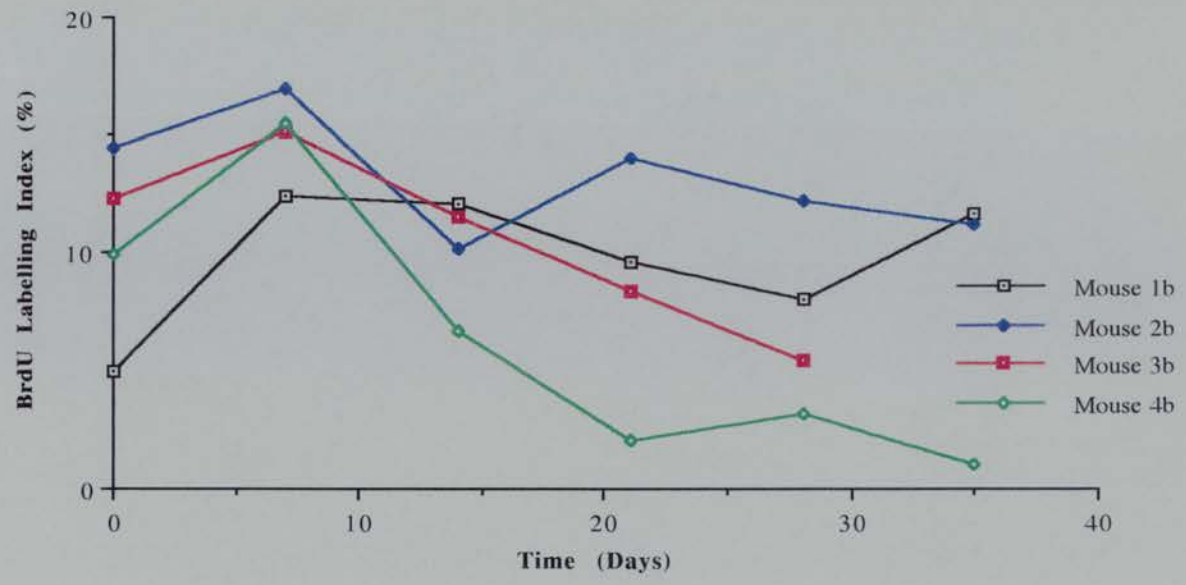
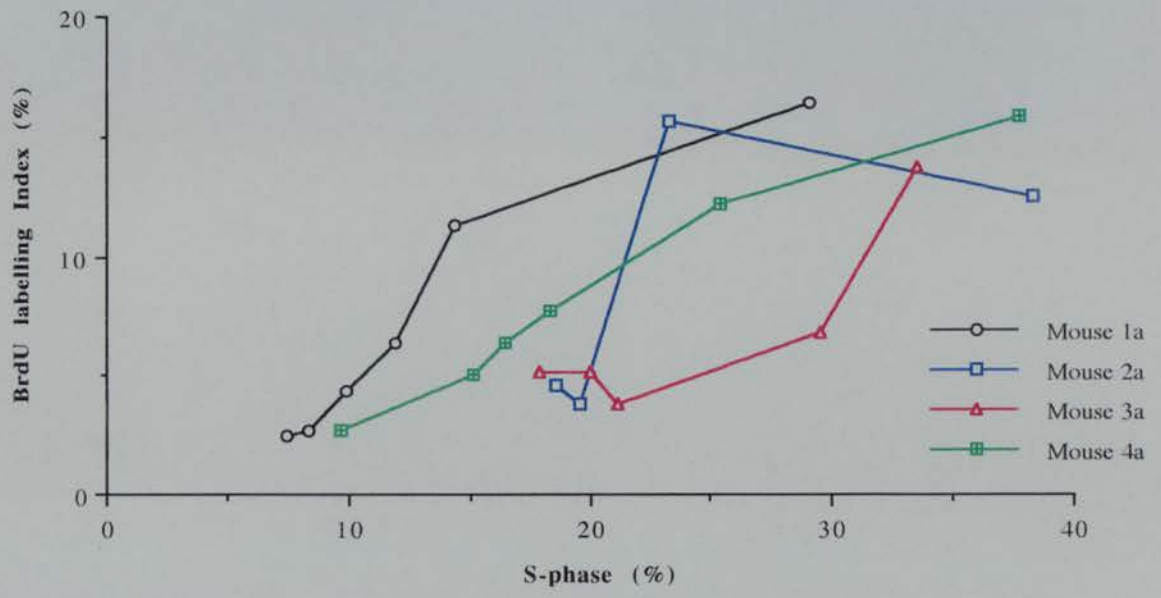


Figure 11.8 shows the variance of the BrdU Labelling index with time in the four adriamycin-treated and the four control T1068 xenografts. The Labelling index decreases with time in both tumour groups although interestingly their appears to be an initial increase over the first 7 days in each tumour in the treated group.

Figure 11.9 The Relationships between BrdU Labelling Index and S-Phase Fraction in Individual T1068 Xenografts; a) Controls and b) Treated with 0.5mg/kg Adriamycin

a



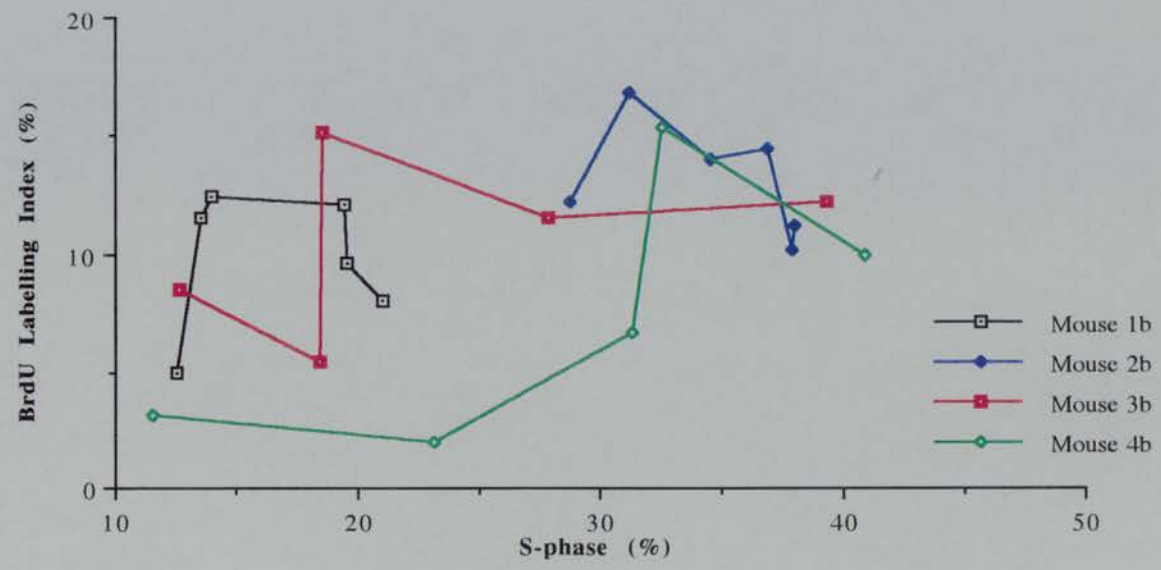
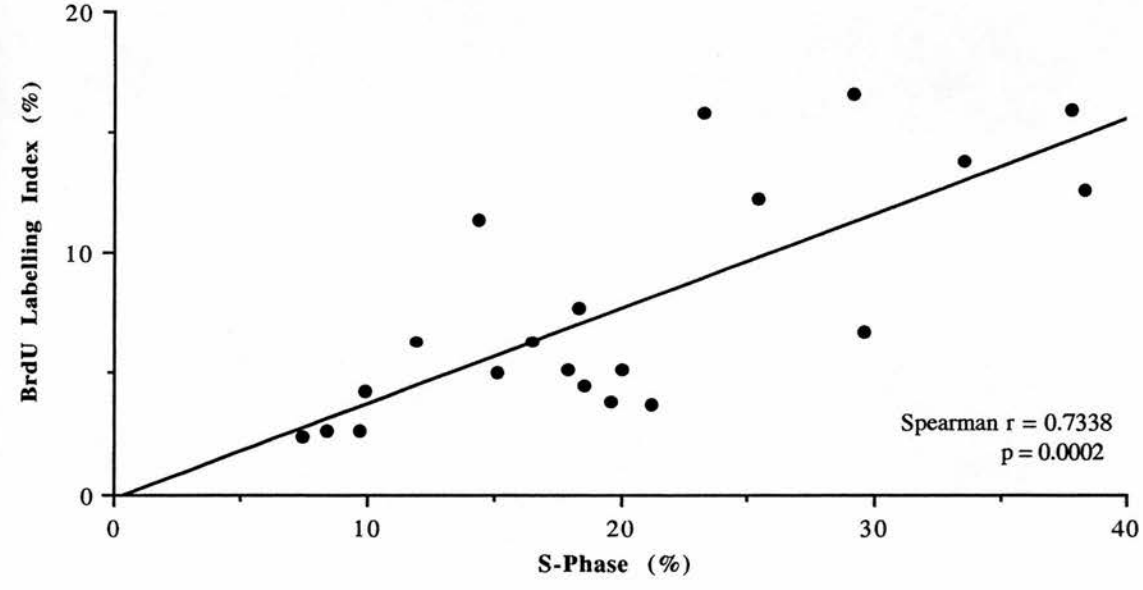


Figure 11.9 shows the relationship between the two markers of proliferation, S-phase and BrdU L.I. for individual tumours sampled serially by Fine Needle Aspiration. Individual control tumours display a relatively good correlation whereas treated tumours do not. This is further demonstrated in Figure 11.12.

Figure 11.10 The Relationship Between BrdU Labelling Index and S-Phase; Combined Values from Serial Measurements in a) Control and b) Treated T1068 Xenografts

a



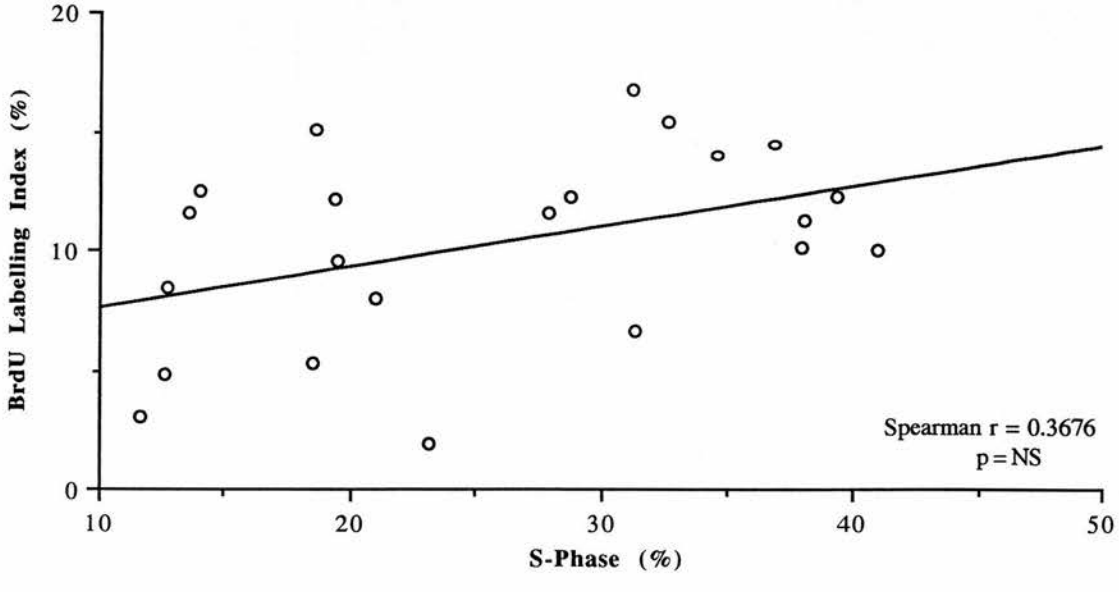


Figure 11.10 shows the relationship between S-phase and BrdU in a) control tumours and b) tumours treated with 0.5mg/kg adriamycin. The points shown represent all the individual samples taken from each of four tumours where a value for both S-phase and BrdU L.I. could be obtained by dual staining. There is a significant relationship in the control tumours but not in those which which have been treated.

Chapter 12: Patient Samples

12.1 Markers of Proliferation

Four possible methods for determining cellular proliferation have been described above in cell lines grown in tissue culture. Having thus tested the methodology an assessment was needed to determine if the same approaches could be used to derive measurements of cellular proliferation in material sampled from human tumours. Fine needle aspiration is a minimally invasive technique for obtaining cellular material from solid tumours but it was unclear whether such a technique could produce sufficient good quality material for several flow cytometric assays.To address these questions, therefore, fine needle aspirates were taken from surgical specimens obtained from an unselected group of 38 patients. All patients had presented with a clinically palpable primary breast cancer and had undergone definitive local regional surgery comprising either a wide local excision or a mastectomy. Aspirates from the tumours were taken immediately after surgical removal and the specimens incorporated with BrdU before snap freezing in liquid nitrogen. The specimens were then stored at -80^{0}C prior to staining and flow cytometric analysis for BrdU, Ki-67 and DNA content.

The results are displayed in Table 12.1 and graphically in Figures 12.1, 12.2 and 12.3. There is a highly significant correlation between S-phase fraction and BrdU labelling index, a similar result to that obtained with the cell line experiments. However unlike the cell line results the correlations between Ki-67 labelling index and both S-phase and BrdU uptake were not significant. This possibly represents, in part, the instability of the Ki-67 antigen on freezing (Dr. R. Camplejohn, Personal Communication) since the cell line work had been carried out with fresh cells.There were doubts, therefore, as to the reliability of Ki-67 antibodies on our particular samples. At the time this work was performed there were no reliable Ki-67 antibodies available for the proposed comparative immunohistochemical studies on formalin-fixed, paraffin-embedded samples, and, therefore, no further work was performed with Ki-67 as a marker of proliferation in clinical samples.

The new antibody, Ki-S1, was examined, at a later date, in a small series of FNA's from 13 unselected primary breast tumours. In similar results to those obtained with Ki-67 there was no significant correlation with S-phase fraction (Figure 12.4). Only eight of these samples had an assessable BrdU labelling index and there was no significant correlation with Ki-S1 labelling index. However, unlike Ki-67, Ki-S1 has been shown to be stable with freezing and is also suitable for immunohistochemical staining of formalin-fixed tissue.

Human tumours, unlike cell lines grown in tissue culture or as xenografts, can be classified by DNA content, broadly, as either diploid, tetraploid, aneuploid or multiploid. DNA histograms obtained from diploid tumours are potentially more difficult to analyse because of the varying contribution of DNA from normal, diploid, stromal cells, and an inability to distinguish between cell types. This is illustrated by the lack of correlation (allbeit in small numbers) between S-phase and BrdU labelling index in the 10 tumours that were classified as diploid and the very good correlation in the 28 non-diploid (tetraploid and aneuploid) tumours (Figure 12.5). There were no significant correlations between either Ki-67 or Ki-S1 and S-phase or BrdU in either diploid or aneuploid populations when assessed individually.

There was a lower rate of cellular proliferation, as determined by both the mean values of S-phase and BrdU uptake, in diploid compared to non-diploid tumours (4.7% vs 9.2% and 2.1% vs 2.4%, respectively). Although these differences are not statistically significant they do reflect previously reported findings as described in chapter 5.2.

The following chapters report results from the two clinical studies examining the clinical response to primary systemic therapy of breast cancer and the associoated biological responses. Based on the preliminary work reported above it was decided to measure both S-phase and *in vitro* BrdU uptake in fine needle aspirates. In addition, in order to compare the efficacy of fine needle aspiration and flow cytometry to standard immunohistochemical techniques as tools for measuring biological response, a further marker that coud be analysed by both techniques was required. The measurement of Ki-S1 expression appeared to fulfil this criteria.

Table 12.1 S-Phase, BrdU and Ki-67 Labelling Indices in Samples Obtained by Fine Needle Aspiration from 38 Human Primary Breast Cancers.

Patient	S-Phase	BrdU L.I.	Ki-67 L.I.	DNA Index
1	0.20	1.59	23.42	1.00
2	7.50	2.42	11.67	2.67
3	13.60	2.44	18.59	1.78
4	7.10	1.53	21.96	1.69
5	7.10	2.15	4.79	2.18
6	3.90	1.02	0.90	1.79
7	12.20	2.52	14.30	1.94
8	1.40	0.34	42.21	2.25
9	28.20	1.31	21.48	2.03
10	22.60	3.45	8.20	1.99
11	12.20	3.80	21.20	1.00
12	7.00	1.87	10.99	1.78
13	6.00	1.41	0.42	1.00
14	2.80	4.53	9.50	1.00
15	11.10	3.63	23.03	2.01
16	3.00	1.01	31.65	1.49
17	0.30	1.23	14.53	1.94
18	4.60	0.30	0.18	1.00
19	4.20	3.39	10.00	1.00
20	2.50	0.81	4.40	1.93
21	2.80	0.93	36.24	1.18
22	19.20	5.65	2.56	1.51
23	10.50	1.75	22.02	1.86
24	16.60	6.10	5.76	1.74
25	12.40	0.87	10.35	1.00
26	19.90	2.31	6.17	1.66
27	10.70	4.59	15.66	1.60
28	2.00	0.72	24.60	2.67
29	8.80	5.23	28.41	1.90
30	13.30	8.46	15.04	1.62
31	3.10	1.50	29.32	1.00

Table 12.1 Contd.

32	7.10	2.97	43.76	1.60
33	0.70	1.30	26.84	1.00
34	0.90	2.52	11.27	1.00
35	3.70	0.28	4.88	1.79
36	1.20	1.80	8.91	1.62
37	4.50	0.75	25.71	2.09
38	9.60	1.07	54.53	2.62

Table 12.1 shows data obtained from flow cytometric measurement of cellular proliferation in samples obtained by fine needle aspiration from the primary tumours of 38 patients with breast cancer. Analysis of DNA histograms also enabled an estimate of DNA index to be made (0.95-1.05 = diploid, 1.95-2.05 = tetraploid and others aneuploid). The relationships between the different parameters are displayed graphically in figures 12.1 to 12.3.

Figure 12.1 The Relationship Between S-Phase and BrdU Labelling Index in Fine Needle Aspirates from 38 Patients with Breast Cancer.

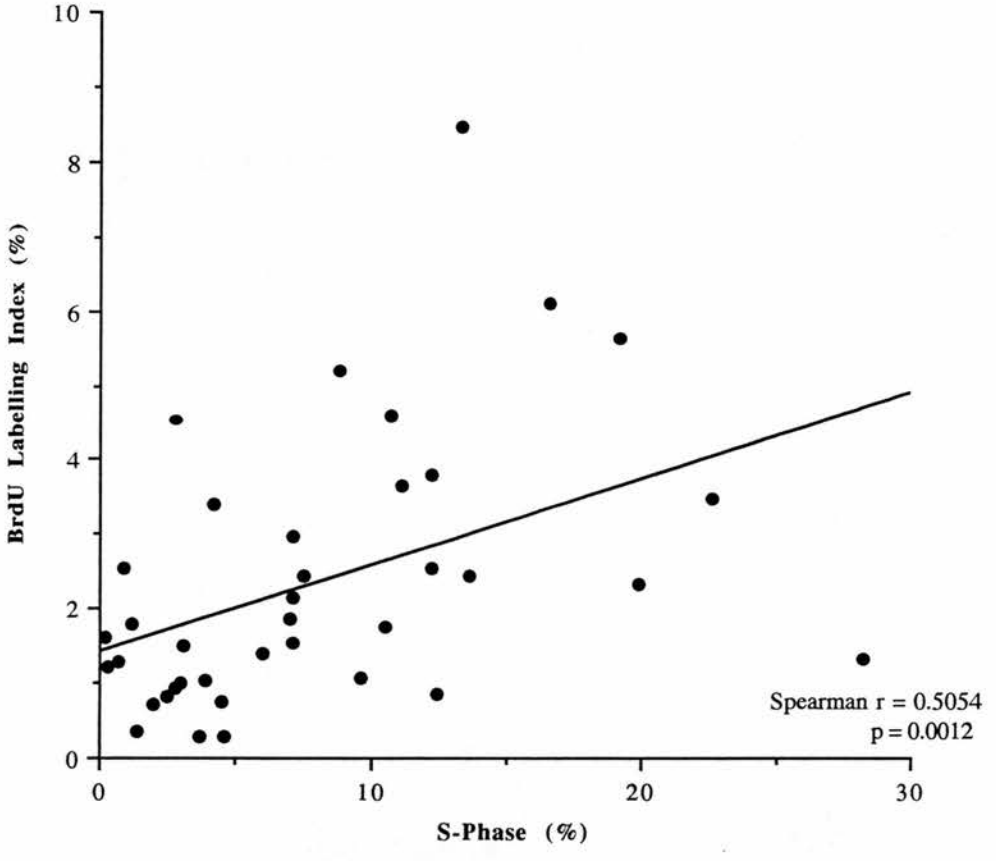


Figure 12.1 shows graphically the correlation between S-phase and BrdU uptake in dual-stained cells obtained by fine needle aspiration from an unselected group of 38 patients with primary breast cancer. There is a significant correlation between these two parameters. This correlation appears to be better in a subset of 28 patients with aneuploid tumours (see fig 12.4)

Figure 12.2 The Relationship Between S-Phase and Ki-67 Labelling Index in Fine Needle Aspirates from 38 Patients with Breast Cancer

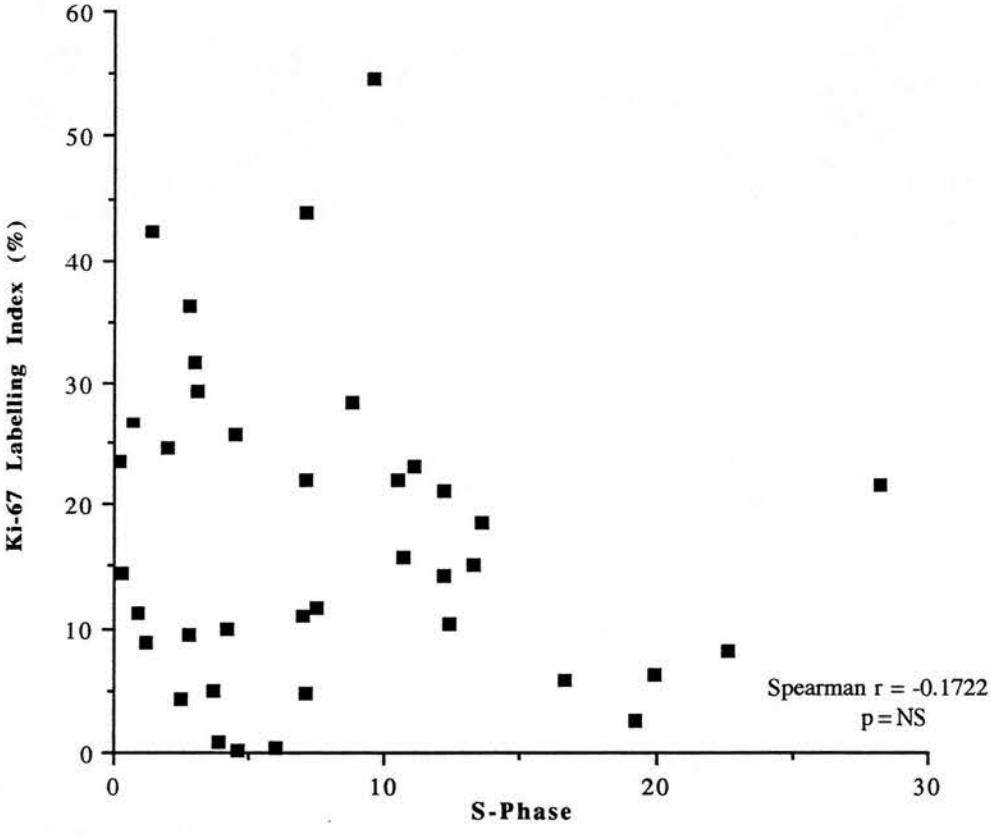


Figure 12.2 shows the relationship between S-phase fraction and Ki-67 Labelling Index, as assessed by flow cytometry, in FNA samples taken from 38 primary breast tumours in a group of unselected patients. There is no significant correlation between these to parameters in this group of samples.

Figure 12.3 The Relationship Between BrdU and Ki-67 Labelling Indeces in Fine Needle Aspirates from 38 Patients with Breast Cancer

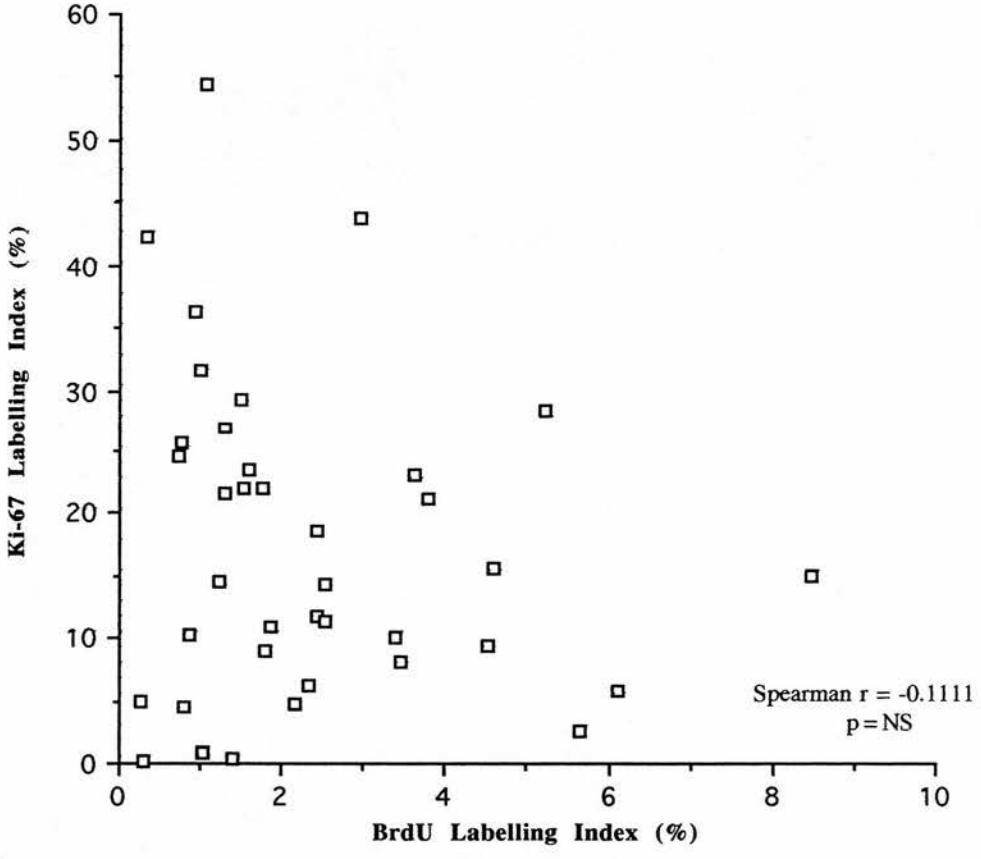


Figure 12.3 shows the relationship between Ki-67 expression and BrdU uptake, as assessed by flow cytometry in a series of FNA's taken from unselected patients with primary breast cancer. There appears to be no significant correlation. This is contrary to the situation in cell lines grown in tissue culture and probably reflects the unreliability of Ki-67 in frozen fresh tissue specimens.

Figure 12.4 The Relationship Between S-Phase and Ki-S1 Labelling Index in Fine Needle Aspirates from 13 Patients with Breast Cancer

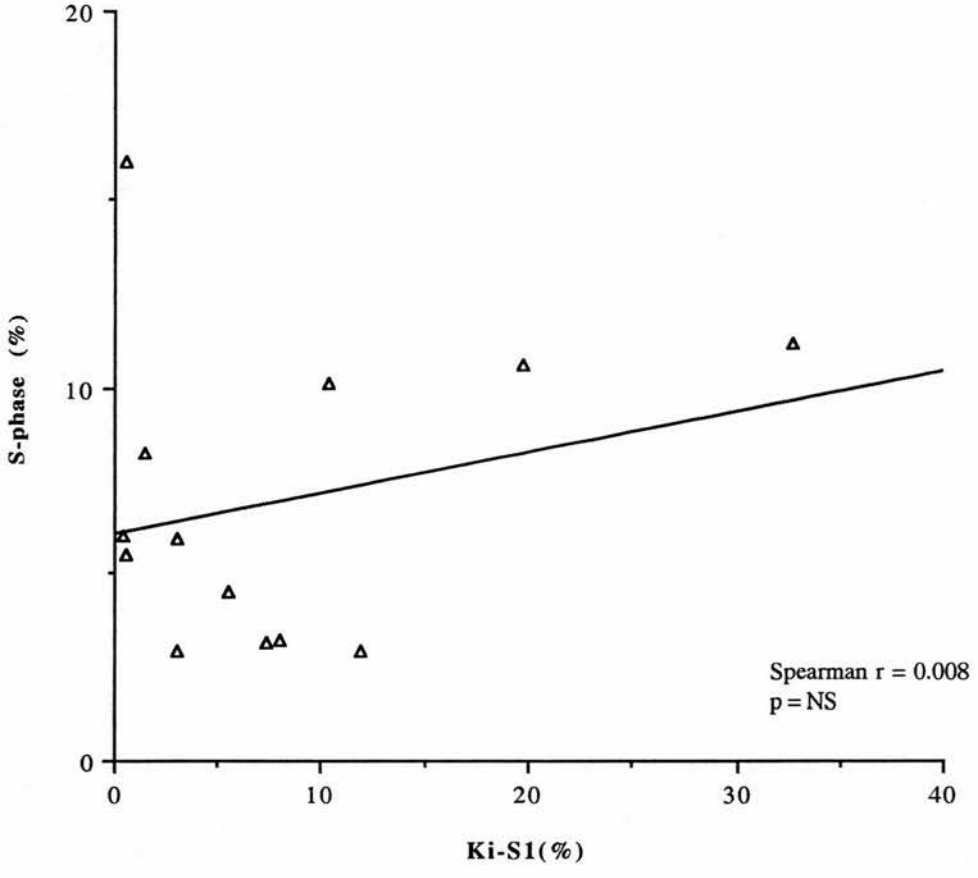


Figure 12.4 shows the relationship between Ki-S1 expression and S-phase fraction in a small group of 13 primary breast tumours. There is no significant correlation between these two parameters in this population.

Figure 12.5 The Relationship Between S-Phase and BrdU Labelling Index in Fine Needle Aspirates from Patients with Breast Cancer in a) Aneuploid and b) Diploid Tumours.

a)

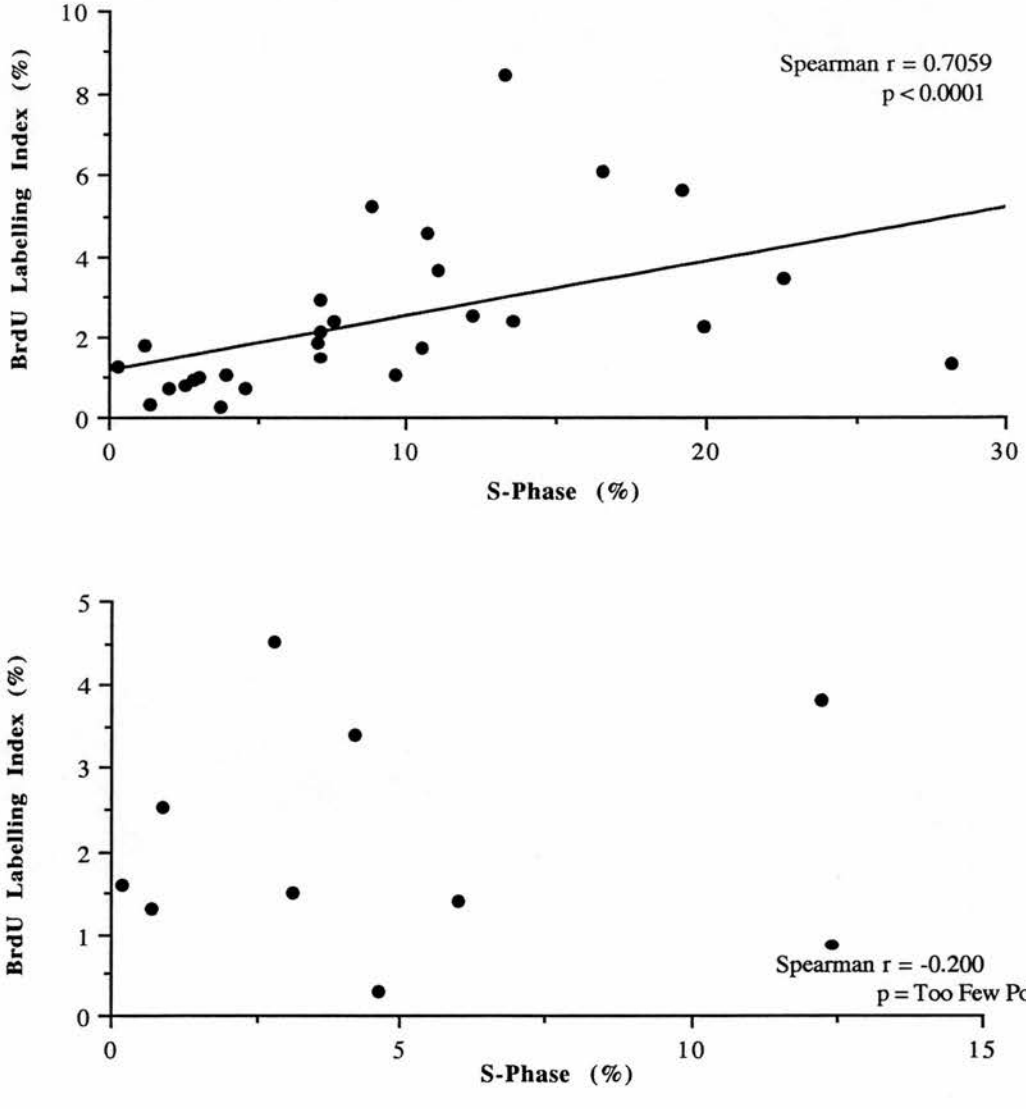


Figure 12.5 shows the correlations between S-phase and BrdU uptake in FNA's from aneuploid (including tetraploid) and diploid tumours. Although numbers are small there is a highly significant correlation in non-diploid as opposed to diploid tumours. This probably reflects the difficulty in determining S-phase in diploid DNA histograms with no method of distinguishing tumour cell DNA from that from "normal" stromal cells.

CHAPTER 13 : Clinical Response to Primary Systemic Therapy

Clinical studies were performed to examine the response of breast tumours to primary sytemic therapy with either chemotherapy or hormonal manipulation. These studies involved the examination of response at a macroscopic level by serial ultrasound measurements and also the consideration of response at a cellular level with immunohistochemical and flow cytometric measurements of biological markers. Most results relate to the group of elderly patients treated with primary tamoxifen because this represented the largest population of patients recruited.

13.1 Ultrasound Measurements

Ultrasound scanning of the breast has previously been shown to be an accurate method of measuring tumour volume as discussed in Chapter 2. To confirm this to be true in the author's hands a series of 30 patients, whose response to tamoxifen was monitored and reported below, were studied. The mean diameter of an individual tumour as measured clinically by calliper or by ultrasound scanning immediately prior to surgery was compared to the diameter measured by the pathologist on cutting the surgical specimen. The mean values for each method of assessment represented the mean of two diameters measured at 90^0 in the same plane. All calliper and ultrasound measurements were performed by the author. The correlations between each method of clinical assessment and the pathological measurement are displayed graphically in figures 13.1 and 13.2. There are significant correlations with pathological diameter for both calliper ($p=0.0003$) and ultrasound ($p<0.0001$) measurements. The correlation between calliper and pathological measurements is the poorer of the two and the graph demonstrates a particularly wide spread of calliper measurements at pathological diameters less than 1.5cm. Small tumours are difficult to measure clinically with any accuracy but such tumours are usually relatively easily identifiable by ultrasound as demonstrated by a good correlation in such measurements with pathological direct assessment. Indeed of the 30 patients studied 7 had tumours that, by the time of surgery, could not be distinctly palpated and measured by calliper although still visible by ultrasound. Figure 13.2 thus represents data from only 23 of the patients studied.There is one obvious ouytlying point in the graph depicting the correlation of ultrasound and pathological tumour measurement and this represents a large lobular carcinoma which are a notoriously difficult histological tumour type to define precisely on ultrasound scanning.

Ultrasound appears, therefore, not only to reflect the actual size of the tumour with

greater accuracy but also to enable measurement and monitoring of the response of those tumours which maybe small and/or deep-seated within the breast. One could assume that the greater accuracy of ultrasound over calliper measurement would also allow a better determination of small and early responses to therapy.

Figure 13.1 The Relationship between Breast Tumour Diameter as Determined by Ultrasound and the Direct Pathological Measurement

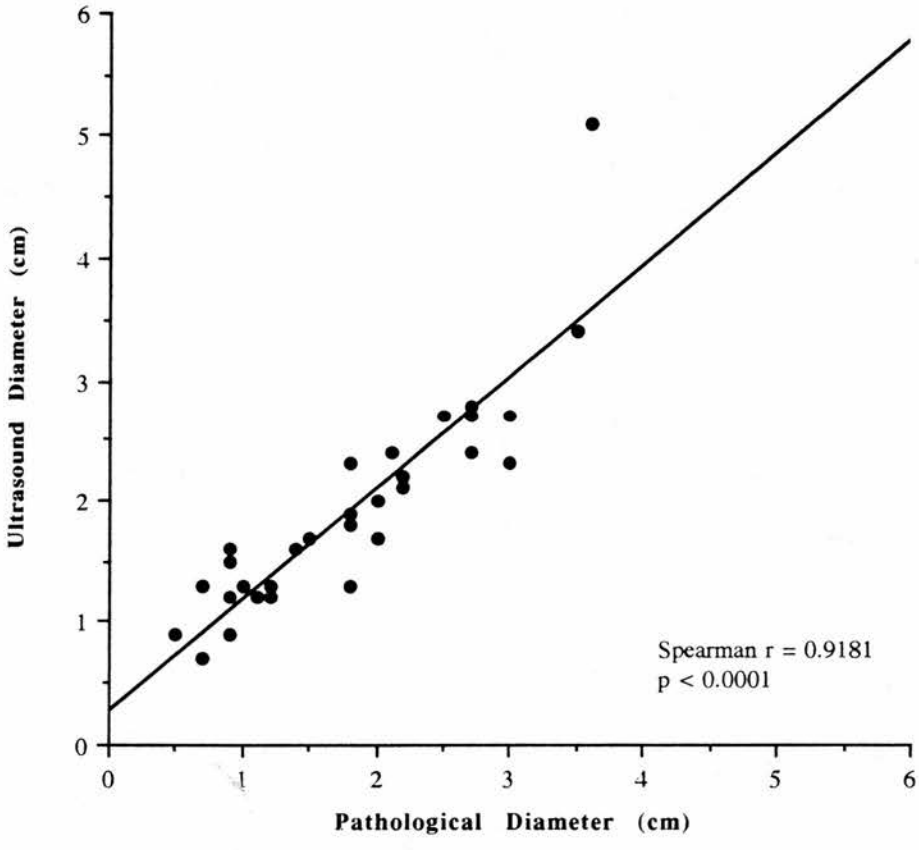


Figure 13.1 shows the correlation, in 30 patients, between the mean tumour diameter as assessed by ultrasound scanning immediately prior to surgery and that determined by direct measurement of the surgical specimen on cutting. A highly significant correlation is represented between pathological measurements of 0.5 to 3.0cm with one outlier at 3.5cm. This point represents a large lobular carcinoma which are notoriously difficult to define clearly on ultrasound scanning.

Figure 13.2 The Relationship between Breast Tumour Diameter as Determined Clinically by Calliper and Direct Pathological Measurement

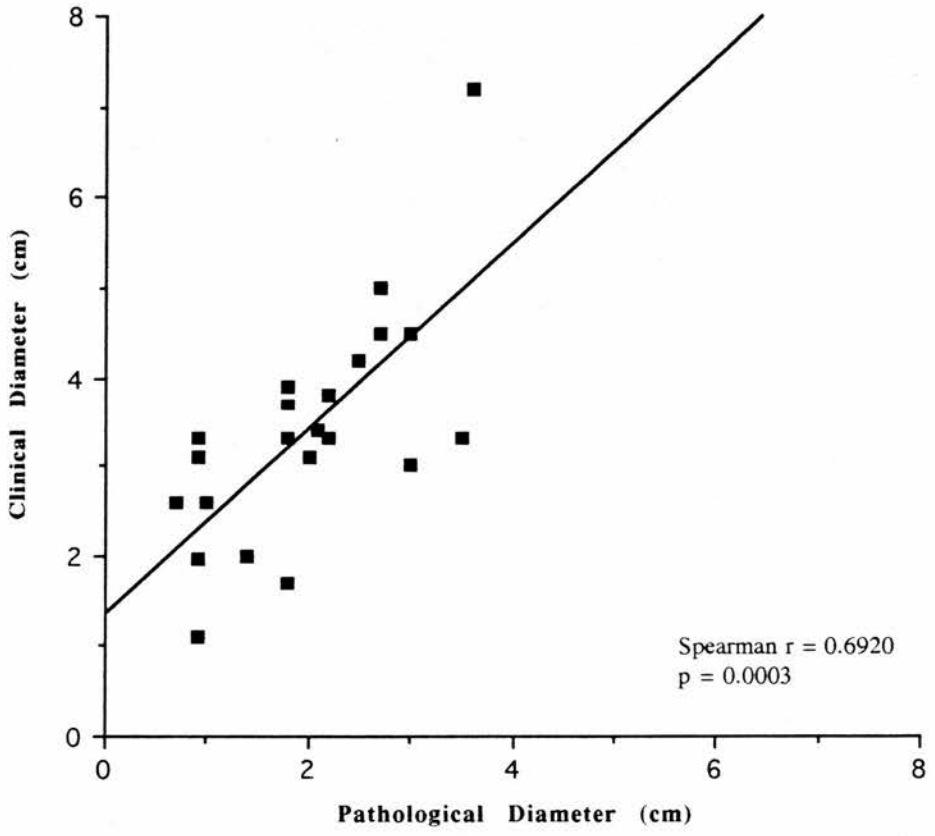


Figure 13.2 shows the correlation, in 23 patients, between the mean tumour diameter as determined clinically, by calliper measurement and the direct pathological measurement of the cut surgical specimen. There is a significant correlation between the two methods of assessment although at pathological diameters below 1.5cm there is a wide spread of corresponding clinical measurements. Data is represented from only 23 of the study population of 30 patients as tumours in 7 patients could not be clinically palpated or clearly defined for calliper measurement. The gradient and intercept of the line of "best-fit" demonstrate the consistently greater diameter measured clinically than that seen pathologically which is accounted for by the skin and subcutaneous tissues included in calliper measurements.

13.2 The Clinical Response to Tamoxifen in Elderly Patients

In order to examine the early tumour response to systemic therapy with tamoxifen a group of 94 patients were assessed. All patients were over the age of 70 and had presented with primary breast tumours. One patient presented with bilateral disease and the response of each individual tumour was monitored and recorded. The mean age of patients within the study was 76.1 years with a range of 70 to 87 years of age. All patients were free of metastatic disease as determined by standard staging investigations. Oestrogen receptor levels were measured by enzyme immunoassay (EIA) of pre-treatment wedge biopsies and/or immunocytochemical assay (ER-ICA) of pre-treatment fine needle aspirates. Adequate specimens for analysis were not obtained in three patients (these three patients went on to receive tamoxifen despite unknown ER status because of a wish not to undergo surgery and all demonstrated a subsequent response). All other patients had ER +ve tumours, with receptor levels of >20fmol/mg cytosol protein, as determined by EIA, or positive staining in >10% cells by ER-ICA.

Tumour volumes were assessed by ultrasound scanning prior to the commencement of treatment and at monthly intervals thereafter. All patients were monitored for at least three months, with 26 patients monitored up to six months. The responses in individual tumours were recorded as the percentage reduction in ultrasound assessed volume, with respect to the pre-treatment value (Table 13.1). A response (>10% reduction in tumour volume) was recorded in 70 of the 95 tumours (74%) after three months of treatment. Of these responding patients the mean decrease in tumour volume was 58.5% (range: 16.8 - 91.0%). The distribution of the degree of response after one, two and three months are shown in Table 13.2 and Figures 13.3 to 13.6. A typical series of ultrasound scans, and mammograms for comparison, from the same responding patient are shown in figures 13.7 and 13.8. After one month 44% of tumours demonstrated a response with the remainder showing no change in volume. The majority of responders at this very early time point show a 10-50% reduction in tumour volume but one tumour appeared to be extremely sensitive demonstrating a reduction of >75%. No tumour increased in size after one month. By two months 64% had demonstrated a response with a much greater proportion having volume reductions of >50%. By this time point four tumours had increased in size. After three months 70 tumours had reduced in size with 19 (27%) displaying a reduction in tumour volume of >75%. These figures can be illustrated practically in that sufficient reductions in tumour volumes may enable the avoidance of mastectomy. Of 26 responding patients who went on to definitive local surgery after primary tamoxifen treatment and would

have required a mastectomy at presentation (tumours >4cm in diameter) 10 were treated with a wide local excision. In addition nine of 17 tumours staged as T4 at presentation became operable with treatment. Interestingly the one patient in whom bilateral tumours were monitored showed different responses with one tumour demonstrating a 66% reduction in volume whereas the other remained static with three months of treatment.

Of the 26 tumours were monitored up to six months 20 had shown a response at three months and had been randomised to continue treatment with tamoxifen alone in the protocol of the "Elderly Patients Study". Of these, 14 went on to demonstrate a further decrease in tumour volume (an additional mean reduction of 20.1%) and one tumour showed no further change after the initial response. However five tumours that had initially responded showed an increase in volume between three and six months. Of the six tumours that had shown no response at three months, two increased in size by five months and the remaining four did not change in volume.

As a comparison with conventional methods of response assessment, the tumours have also been graded by U.I.C.C. response criteria, based on serial calliper measurements, after three months of therapy (Table 13.2). Calliper measurements were available for 80 of the 95 tumours studied (data not shown). Of these tumours 27 (34%) were classified as responders (19 partial and 8 complete responders) as compared with 60 (75%) of the same population as assessed by ultrasound. The range of values obtained for the percentage change in tumour volume, as assessed by ultrasound, in each of the groups selected according to UICC response is shown in Figure 13.9. Although there were statistically significant differences between the mean redution in ultrasound assessed volumes in tumours classified as either "no-change", "partial response" or "complete response" there was a degree of overlap. This was particularly noted in the "no change" group with some tumours demonstrating reductions in volume of over 80% as measured by ultrasound. Thus, there was no clear "cut-off" in ultrasound assessed tumour reduction at which point all tumours were also classified as responders by UICC criteria.

Ultrasound has enabled us to monitor accurately the early responses to tamoxifen and to our knowledge this is the first time such data has been reported. After three months of treatment the data suggest that all those tumours that will respond to tamoxifen have demonstrated a volume reduction. The numbers of non responding patients monitored to six months is, however, relatively small. There is clearly great potential in primary treatment with tamoxifen in the elderly population to downgrade the operation required for definitive local surgery and to enable inoperable locally advanced tumours to become operable. The associations that were observed in this group of 94 patients

between overall response / degree of response and the clinical characteristics of the tumours (including oestrogen receptor data) are described below.

Table 13.1 The Response to Primary Tamoxifen Therapy in 94 Patients of 70 Years of Age or Over

Patient	Age	Stage	Clin Diam	LnVol (USS)	E.R.	ERICA	1 Month	2 Months	3 Months	6 Months	U.I.C.C. 3/12
1	78	T2N0MO	4.6	2.58	860		0.0	0.0	41.0	72.0	nc
2	72	T2N0M0	3.3	1.34	606		0.0	0.0	68.7		pr
3	80	T2N1M0	4.2	2.25	1491	41	30.0	55.0	72.0		pr
4	78	T2N0M0	2.8	1.33		22	0.0	48.3	24.9		nc
5	71	T2N0M0	3.4	1.40	325	33	0.0	35.0	54.0		nc
6	74	T2N0M0	3.8	3.54	29	10	0.0	0.0	0.0		nc
7	80	T2N0M0	3.6	2.20	425		0.0	59.0	69.9		nc
8	83	T4bN1M0	4.2	2.63		34	0.0	0.0	16.8	38.4	nc
9	70	T3N1M0	7.0	3.77	86	50	0.0	0.0	0.0		nc
10	71	T1N0M0	1.9	0.86		51	30.0	62.0	73.0	77.0	cr
11	85	T4bN1M0	5.8	3.01	605	55	0.0	28.0	48.0		pr
12	73	T2N0M0	2.5	1.55		60	46.7	58.2	69.6		*
13	70	T2N0M0	4.6	2.26	444	34	76.3	87.8	87.7		pr
14	70	T2N0M0	3.4	1.89			0.0	0.0	33.5	54.0	nc
15	72	T2N0M0	4.0	1.73	388		0.0	0.0	50.7		pr
16	71	T4bN1M0	4.3	2.60	87	40	0.0	0.0	0.0	prog 5/12	nc
17	83	T2N0M0	2.1	0.87		62	0.0	0.0	0.0		nc
18	70	T2N0M0	3.9	2.81		83	65.0	91.0	90.5		cr
19	80	T2N0M0	3.6	2.30		67	60.1	62.0	64.7	77.5	nc
20	72	T2N0M0	3.7	2.10	79	55	0.0	0.0	23.0		nc
21	71	T2N0M0	3.1	0.99	445	83	39.0	82.0	91.0		cr
22	79	T2N0M0	2.7	1.67		74	42.5	53.0	76.6		pr
23	73	T2N0M0	3.3	0.99	485	70	51.0	87.0	86.0	91.0	cr
24	78	T2N1M0	3.9	2.26	170	31	0.0	0.0	0.0		nc
25	75	T2N0M0	1.9	-0.10		84	0.0	-50.0	-48.0		*
26	78	T2N0M0	4.1	1.60	68	17	0.0	0.0	18.0		nc
27	83	T4aN1M0	2.9	1.44	70		0.0	0.0	0.0	0.0	nc
28	83	T2N1M0	2.8	1.18		29	0.0	10.8	50.4	67.6	nc
29	72	T2N1M0	3.3	1.34	384	22	40.0	34.0	68.0		*
30	83	T2N0M0	4.3	2.21	651	76	18.0	51.3	84.5		**
31	80	T2N1M0	3.7	3.70	24	0	0.0	0.0	0.0		nc
32	73	T3N2M0	5.9	1.94	498	8	0.0	58.6	63.2		**
33	87	T2N0M0	2.0	0.48	442	65	0.0	0.0	25.0		nc
34	71	T2N0M0	3.1	1.52	186	37	0.0	28.0	53.0		pr
35	71	T2N0M0	4.0	1.75	510		0.0	28.7	44.8		nc
36	70	T2N0M0	4.4	2.38	150		0.0	49.9	29.3		nc
37	80	T1N0M0	1.2	-0.80		26	20.0	23.0	24.5		nc
38	72	T1N1M0	2.2	0.05		20	0.0	0.0	0.0		nc
39	71	T3N1M0	5.8	2.47	222	58	0.0	0.0	0.0		nc
40	72	T4bN1M0	4.0	2.35	72	38	18.8	26.9	37.0	78.3	pr
41		T3N0M0	4.9	3.30		46	51.3	61.2	65.2		nc
42	74	T3N1M0	5.3	3.20		23	0.0	0.0	0.0		nc
43	71	T2N1M0	3.9	2.23	93	41	0.0	21.7	33.6		**
44	74	T2N0M0	3.0	1.76	964	69	0.0	62.0	67.0		pr
45	82	T3N0M0	5.3	2.86		80	21.0	45.5	34.6	37.6	nc
46	70	T4bN1M0	6.0	3.17	138	26	18.4	16.2	22.9		nc
47	81	T2N0M0	4.4	1.49	262	61	17.3	28.7	29.1		nc
48	71	T2N1M0	4.0	2.66	463	42	0.0	38.0	49.0		**

Table 13.1 Continued

Patient	Age	Stage	Clin Diam	LnVol (USS)	E.R.	ERICA	1 Month	2 Months	3 Months	6 Months	U.I.C.C. 3/12
49	76	T2N1M0	4.1	2.29	837	44	0.0	35.6	80.1		nc
50	82	T2N1M0	3.0	1.02	519		53.1	71.2	84.5	84.0	*
51	85	T2N0M0	4.0	2.27	70	80	0.0	0.0	0.0	0.0	nc
52	81	T2N1M0	4.9	2.19	294		0.0	0.0	7.0		nc
53	71	T2N0M0	3.6	1.58	99	16	35.5	-23.8	-71.0		**
54	85	T2N1M0	4.5	2.27		95	0.0	70.0	64.0		nc
55	76	T4bN0M0	3.7	1.98	143	24	0.0	0.0	0.0	0.0	nc
56	71	T2N1M0	4.0	1.55	384		0.0	0.0	0.0		nc
57	72	T2N1M0	4.9	2.78	469	32	52.0	71.0	88.0		pr
58	71	T4bN1M0	5.4	3.15	557	23	30.1	55.0	71.9		pr
59	71	T2N0M0	2.0	1.20		27	0.0	0.0	-17.0		prog
60	71	T2N0M0	3.0	2.01		40	68.0	67.2	65.4	61.4	nc
61	79	T2N0M0	4.4	2.08			34.0	55.0	48.0	62.4	nc
62	84	T4bN1M0	4.0	1.80		59	15.0	33.7	40.4	61.0	nc
63	76	T2N0M0	2.5	0.57		65	0.0	16.5	77.5		cr
64	82	T4bN0M0	5.5	2.37	97		0.0	37.6	67.0	57.4	pr
65	73	T2N0M0	3.8	1.67	76	6	0.0	10.9	31.9		nc
66	83	T4bN1M0	3.6	2.01	285	25	23.0	47.7	37.8		nc
67	78	T3N2M0	5.1	2.84	1292	98	17.0	62.0	85.0		pr
68L	74	T2N1M0	5.0	3.49	371	83	0.0	60.0	66.0		**
68R		T2N1M0	3.0	1.11	44		0.0	0.0	0.0		**
69	77	T2N0M0	2.6	2.15	426		0.0	28.9	83.0	51.0	pr
70	70	T4bN0M0	2.8	1.57	177	70	42.5	63.9	81.7		nc
71	70	T4bN0M0	5.7	3.41	65	33	0.0	0.0	0.0	0.0	nc
72	76	T2N0M0	4.1	2.42	91	22	15.0	24.7	61.0		**
73	72	T4bN2M0	8.4	2.33		72	25.0	29.0	89.0	52.0	pr
74	71	T2N0M0	2.2	0.19		17	39.0	41.0	41.0	89.0	pr
75	78	T2N0M0	3.9	1.81	166	61	0.0	30.1	58.9		pr
76	76	T2N1M0	4.4	0.87		27	32.3	38.3	28.6	-16.8	nc
77	87	T2N0M0	5.0	1.76			26.6	60.9	74.8		pr
78	78	T2N0M0	4.1	2.34	265		32.8	-20.3	-116.0		**
79	76	T4bN1M0	3.7	1.07	1496	41	0.0	42.0	68.0		nc
80	79	T4bN1M0	6.3	2.79	279	69	0.0	0.0	24.9	45.8	nc
81	80	T2N1M0	4.8	2.74	73	28	0.0	0.0	0.0		nc
82	85	T2N0M0	3.1	1.91	770	64	20.0	55.0	77.0	90.0	nc
83	75	T2N1M0	3.8	1.82	306	67	48.0	56.0	61.0		nc
84	78	T2N0M0	3.4	0.52	1388	83	55.0	61.0	76.0		cr
85	77	T2N0M0	5.0	2.99	40		0.0	0.0	0.0	prog at 4/12	nc
86	71	T2N0M0	4.9	2.18	201		0.0	0.0	-36.0		nc
87	85	T2N1M0	4.1	1.99	181	13	0.0	-49.0	-72.0		nc
88	78	T2N0M0	3.3	1.32	557	67	30.0	75.0	83.0		cr
89	75	T1N0M0	1.5	0.23		56	40.0	59.0	80.0		cr
90	74	T1N1M0	1.5	1.11		38	21.0	40.1	65.1		nc
91	76	T2N1M0	2.4	1.32		47	19.0	40.2	40.1		**
92	85	T2N0M0	3.3	1.71	161	38	16.0	30.0	74.0		nc
93	70	T4bN1M0	3.5	2.08	120	40	35.9	81.4	81.4		pr
94	75	T2N1M0	5.0	2.60	100	40	0.0	0.0	-16.0		**

Table 13.1 shows data accrued from 94 patients presenting with 95 breast tumours and receiving primary therapy with tamoxifen for at least three months. 91 patients were deemed oestrogen receptor positive by either EIA on pre-treatment biopsies or ER-ICA on pre-treatment FNA's. The remaining three patients were treated despite unknown ER status. Clinical Diameter was assessed by calliper measurement and is expressed in cm. Ultrasound measurements (in cm.) were made in three planes and calculated volumes expressed as the ln. ER levels as determined by EIA are expressed in fmol/mg cytosol protein and ER-ICA results are expressed as % of cells staining. Tumour response at one,two ,three and six months are expressed as a % decrease with respect to the pre-treatment volume. An increase in tumour volume is denoted by a negative value.

* Tumours became diffuse and not accurately measurable.

** Tumours for which some clinical measurements not available.

Table 13.2 The Degree of Response Measured After One, Two and Three Months of Primary Tamoxifen

	Non-Responders		Responders % Reduction in Tumour Volume			
	Increase	Static	10-25%	25-50%	50-75%	>75%
1 Month	0	53	14	19	8	1
2 Months	4	30	7	24	24	6
3 Months	7	18	7	19	25	19

Table 13.2 shows the numbers of patients, at monthly intervals from the commencement of tamoxifen, that can be grouped in various response categories. Non- responders either show a >10% increase in tumour volume or are defined as static (<10% increase or decrease in volume). Responders are grouped according to the % tumour volume reduction. There are increased numbers of non-responders showing an increase in tumour volume by three months and also by this time point an increased number of responders showing volume reductions of > 75%.

Figure 13.3 The Response to Tamoxifen After One Month of Treatment

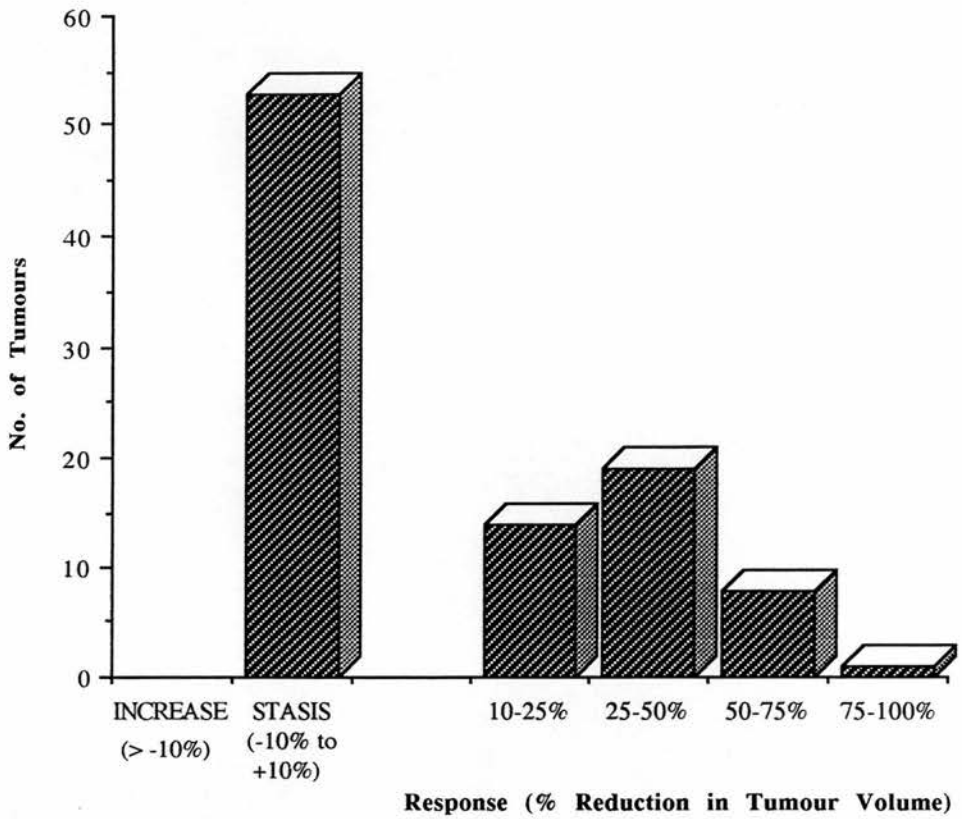


Figure 13.3 shows the distribution of tumour responses at one month. The majority of tumours have shown no change in volume although one tumour has decreased by greater than 75%.

Figure 13.4 The Response to Tamoxifen After Two Months of Treatment

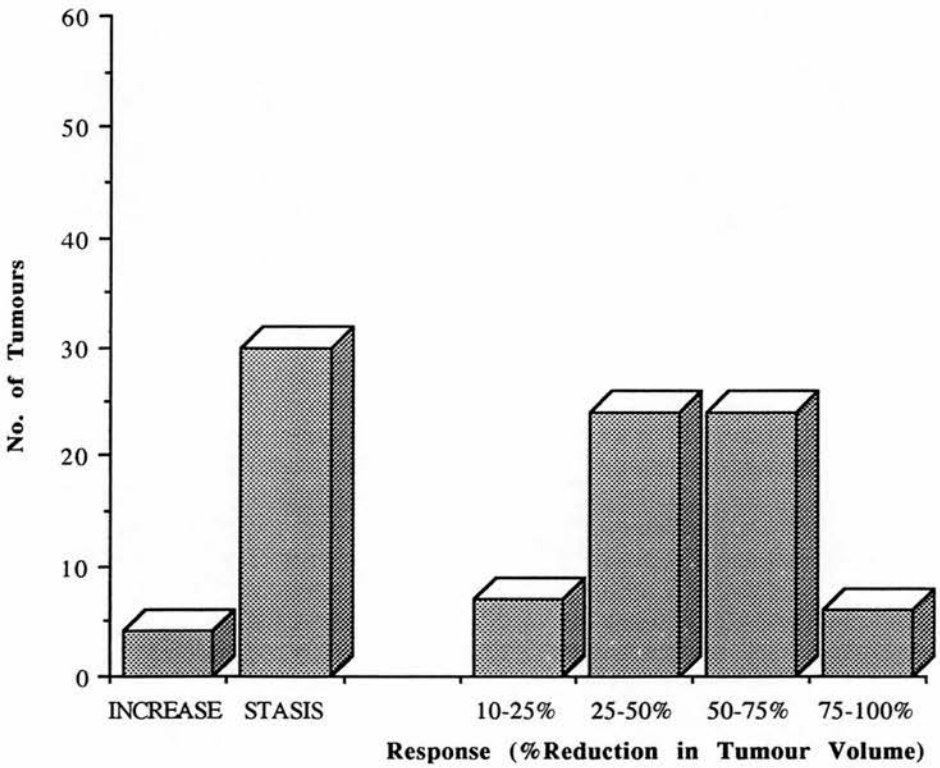


Figure 13.4 shows the distribution of responses after two months of tamoxifen treatment. Four tumours have shown an increase in size and the majority of responding tumours have decreased in volume by greater than 25%.

Figure 13.5 The Response to Tamoxifen After Three Months of Treatment

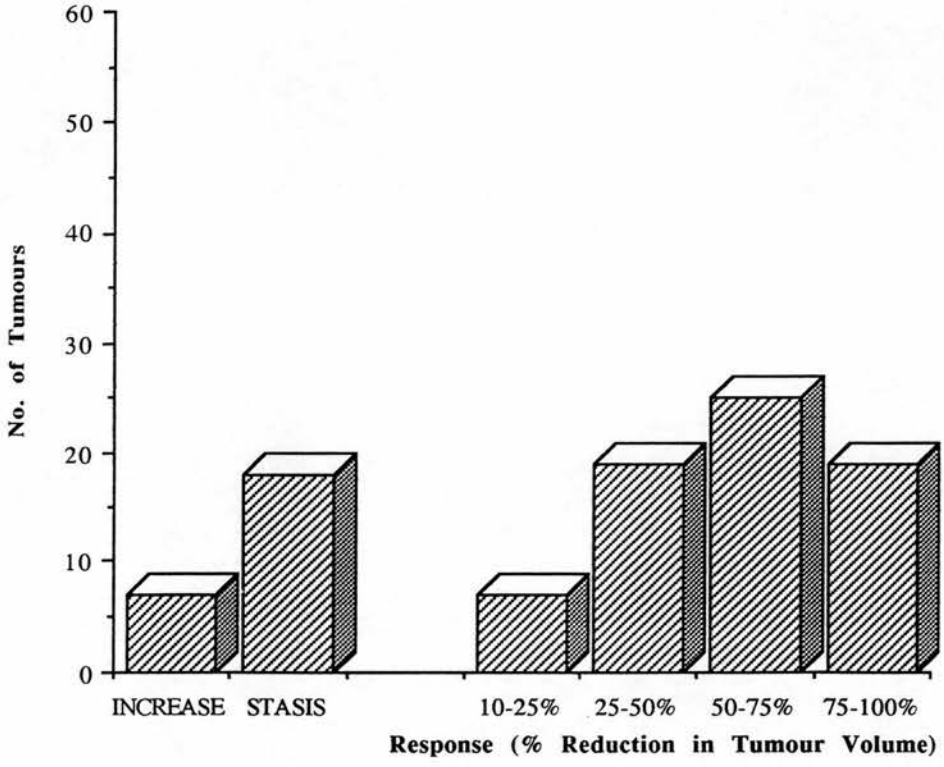


Figure 13.5 shows the distribution of responses observed in a series of 95 tumours after three months of tamoxifen therapy. 70 tumours were classified as responders with the majority displaying >50% reduction in tumour volume.

Figure 13.6 Composite Chart of Responses at One, Two and Three Months of Tamoxifen Treatment

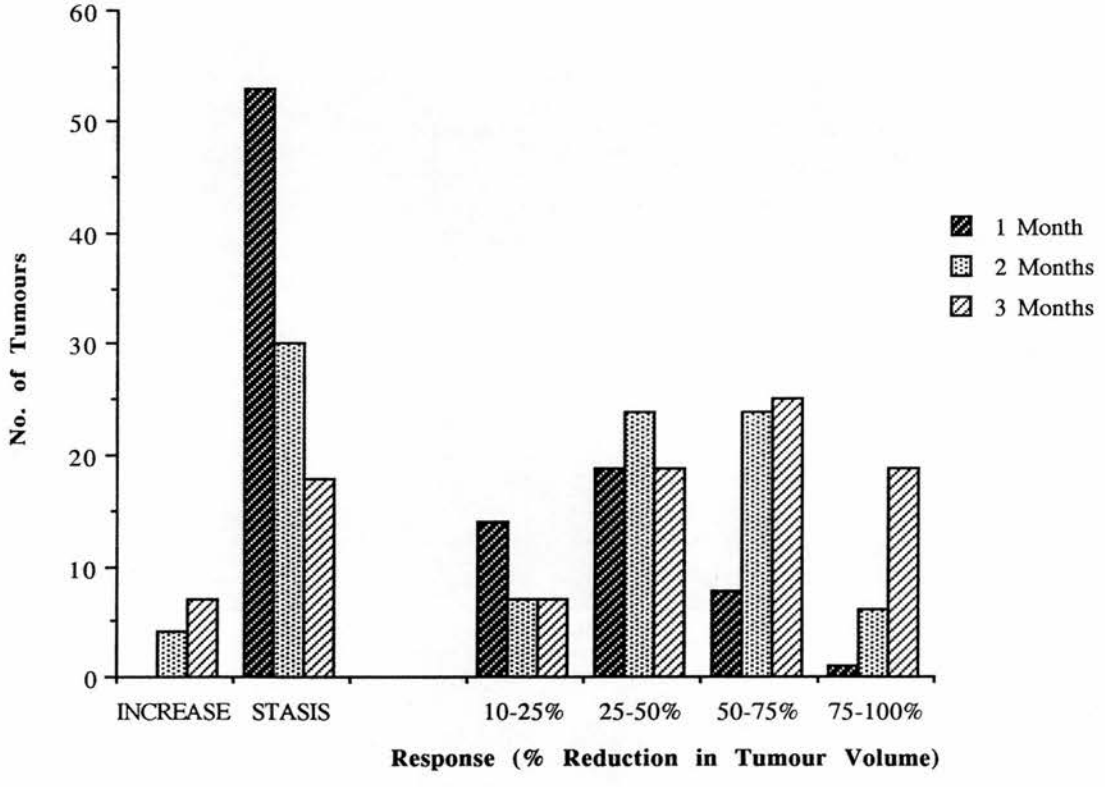


Figure 13.6 shows a composite chart comprising the distributions of tumour responses after one, two and three months of tamoxifen therapy. There are progressively fewer "static" tumours and more displaying either an increase in volume or a greater decrease in volume.

Figure 13.7 Series of Breast Ultrasound Scans Performed at Four-Weekly Intervals on a Patient Responding to Tamoxifen

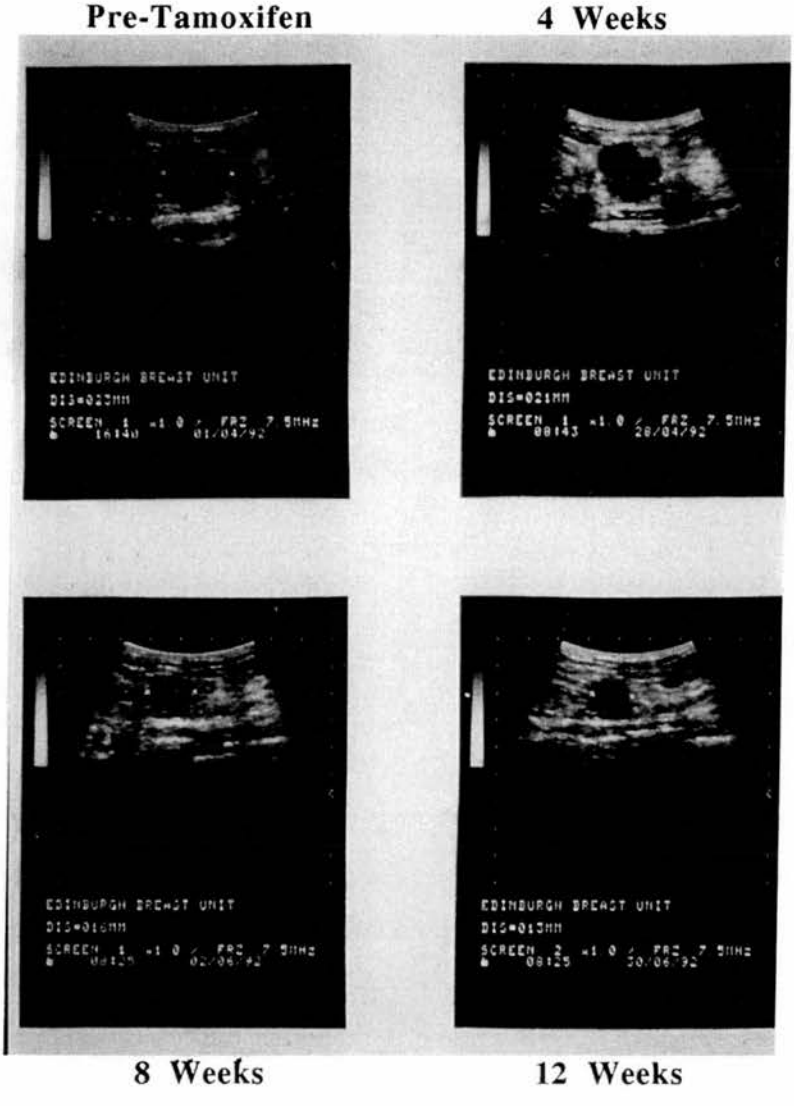


Figure 13.7 shows a series of four ultrasound scans taken before treatment and then at four-weekly intervals thereafter. The tumour is demonstrated as a hypoechoic roughly circular region in the centre of each scan with typical wall "shadows". The tumour diameter, in the single plane view shown, as determined by electronic callipers is recorded below each scan.

Figure 13.8 Mammograms Performed before and after Treatment with Tamoxifen

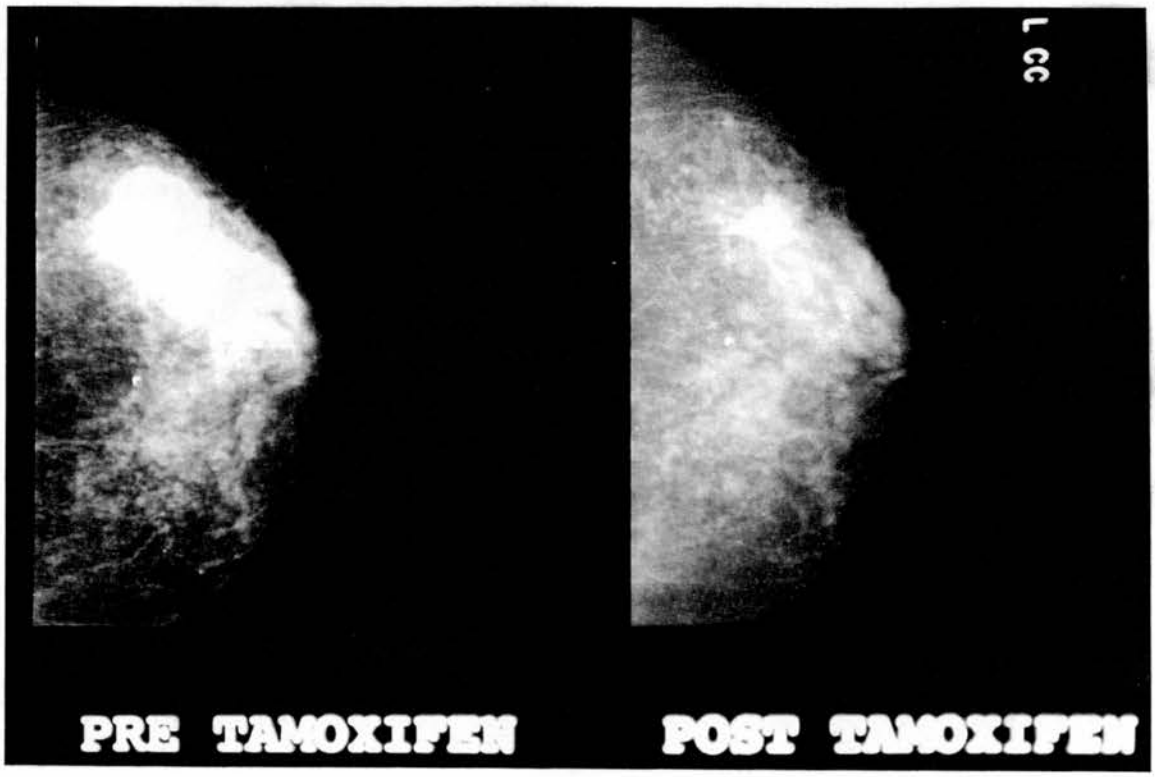


Figure 13.8 shows reproductions of mammograms performed on the same responding patient whose ultrasound scans are shown in Figure 13.7. The X-Rays were performed before and after three months treatment with tamoxifen and demonstrate the comparative ease of tumour measurement by ultrasound.

Figure 13.9 The Relationship Between Tumour Response Graded by UICC Criteria and the Response as Assessed by Ultrasound

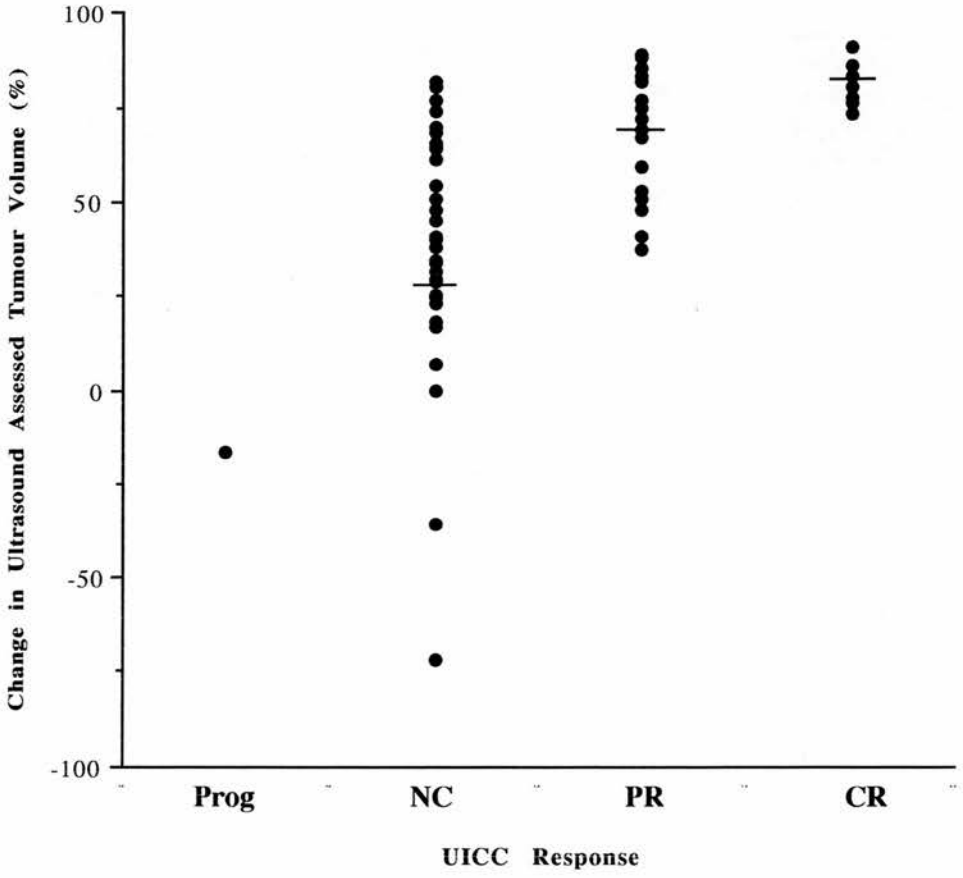


Figure 13.9 shows the relationship between tumour response determined by calliper measurements and then graded by UICC criteria and the response measured by ultrasound scans. There are statistically significant differences between the mean change in ultrasound assessed volume of the groups "NC" and "PR" (Mann-Whitney U-Statistic 120, $p < 0.0001$) and groups "PR" and "CR" (Mann-Whitney U-Statistic 34.5, $p = 0.03$)

Prog = Progression PR = Partial Response
NC = No Change CR = Complete Response

Factors Predicting Response to Tamoxifen

a) Tumour Size

To determine if the size of the primary breast tumour prior to treatment influenced the subsequent observed response the range of values of ultrasound assessed initial volumes were plotted for responders and non-responders (Figure 13.10). There was no significant difference in pre-treatment volume between the two groups. Additionally the initial tumour volume did not relate to the degree of response subsequently observed in responding tumours (Figure 13.11). Grouping tumours into T stages (of the TNM classification and based on calliper measured diameters) also did not produce a significant relationship with response.

b) Lymph Node Status

The presence of axillary lymph node metastases from primary breast cancers has been found to be one of the most consistent markers of poor prognosis. To determine whether the presence of nodal metastases has an association with the response of the primary tumour to tamoxifen, a subgroup of 51 patients who had undergone axillary surgery after primary tamoxifen were studied. Patients were classified as histologically node-negative or positive and these groups contained 26 and 25 patients respectively. Although the mean reduction in volume at three months was lower in the node positive group this failed to reach statistical significance (Figure 13.12).

c) Oestrogen Receptor Levels

All tumours included in the study were deemed ER positive (other than the three tumours discussed above). Two methods for assessing ER levels were employed; EIA on tumour wedge biopsies and ER-ICA on cells obtained by FNA. Results from each of these two methods were available for 50 of the series of 95 tumours and this enabled a direct comparison of their respective predictive values, with respect to tamoxifen response, to be made. ER levels and the relationship to overall response are displayed in Figures 13.13 and 13.14 for assays by EIA and ER-ICA respectively. The median values of ER measurement by both EIA and ER-ICA are significantly higher in responders compared to non responders. There is a greater degree of statistical significance, however, for the data derived from EIA. Perhaps most importantly, and clinically useful, there appears to be a clear cut-off in the EIA data

with all tumours having ER levels of >250fmol/mg of cytosol protein showing a response by three months. There is no such cut-off for ER-ICA estimations. To assess the relationship between ER level as determined by the two methods and the degree of response corresponding values for individual tumours were plotted and Spearmen rank correlations performed (Figures 13.15 and 13.16). There are significant correlations between both EIA and ER-ICA estimations of ER content and the degree of response obseved by ultrasound measurement, but the correlation is of greater statistical significance for the data derived by EIA.

Other biological factors which may predict or reflect the response to tamoxifen are discussed in chapter 14.

Figure 13.10 Pre-Treatment Tumour Volume and the Overall Subsequent Response to Tamoxifen

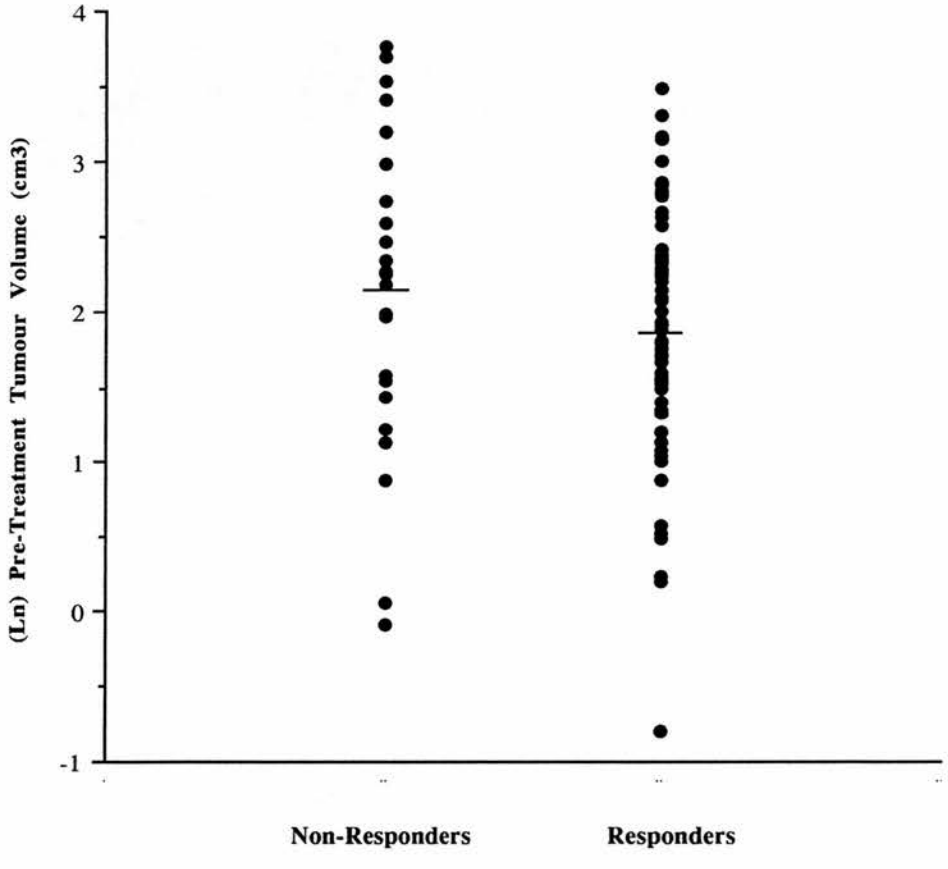


Figure 13.10 shows the relationship, in 95 tumours, between pre-treatment volume, as measured by ultrasound, and the subsequent overall response to tamoxifen after three months of treatment. There is no significant difference between the median volumes of the responding and non-responding groups (Mann-Whitney U-statistic = 687.5, p = 0.1141)

Figure 13.11 Pre-Treatment Tumour Volume and the Degree of Subsequent Response to Tamoxifen

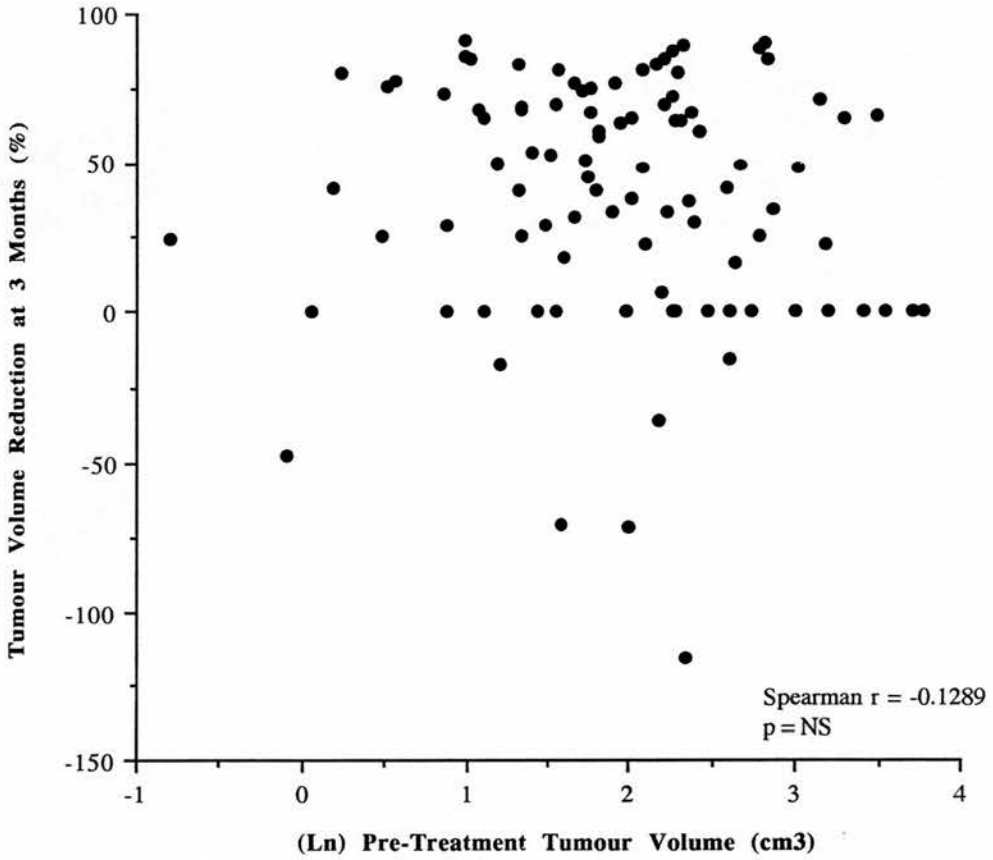


Figure 13.11 shows the relationship between pre-treatment tumour volume as assessed by ultrasound and the degree of subsequent response to tamoxifen after three months therapy. There is no significant correlation between these two variables.

Figure 13.12 The Relationship between Pathological Lymph Node Status and Response to Tamoxifen

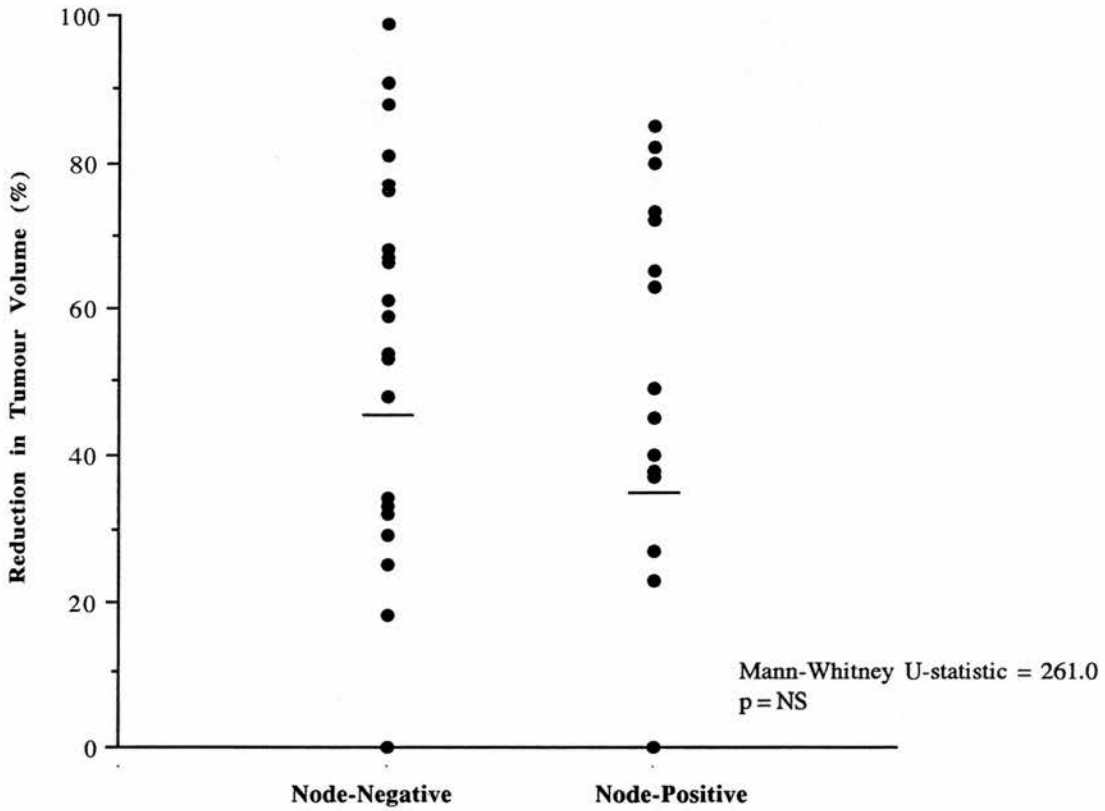


Figure 13.12 shows the relationship between lymph node status and the response to tamoxifen as defined by % reduction in tumour volume. Node status was determined histologically from specimens obtained at the time of definitive loco-regional surgery. There is no statistically significant relationship between these two parameters.

Figure 13.13 Pre-Treatment Oestrogen Receptor Levels as Determined by EIA and Subsequent Overall Response to Tamoxifen.

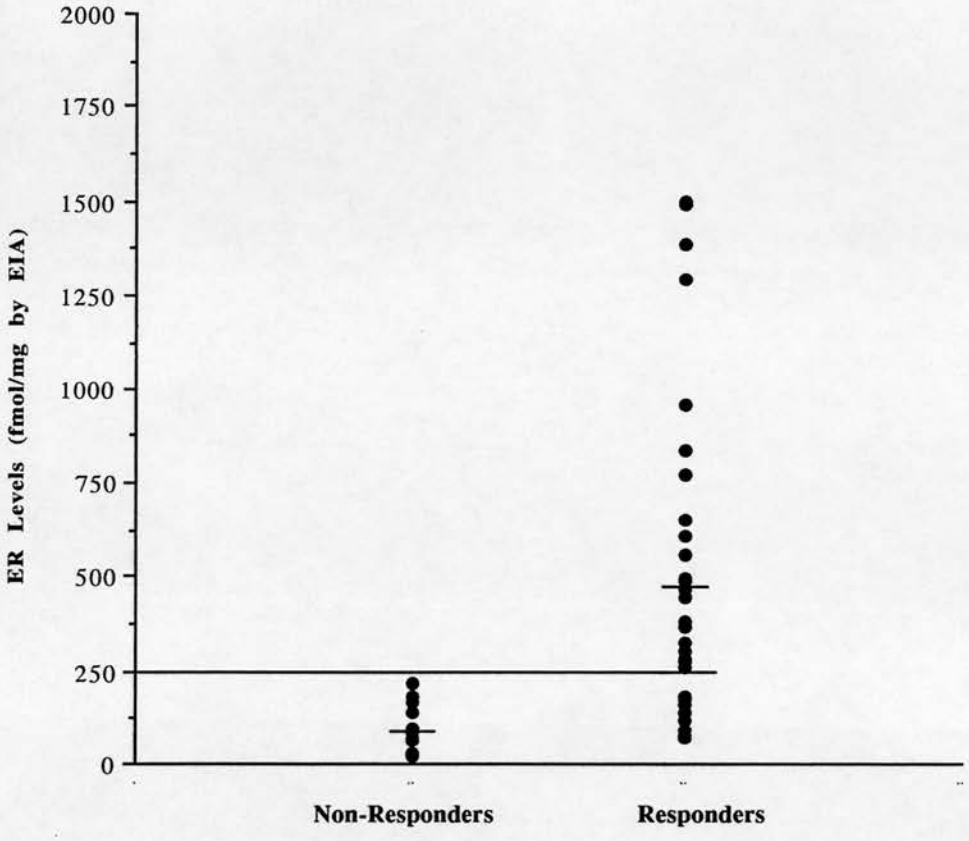


Figure 13.13 shows the relationship between pre-treatment ER levels as determined by EIA and the subsequent overall response to tamoxifen. In this population of 50 tumours (a subset with ER measured by both EIA and ER-ICA) there is a significant difference between median values of ER in responding and non-responding patients (Mann-Whitney U-statistic = 64, p = 0.0001). There is also a clear cut-off in ER values such that all tumours with an ER >250fmol/mg (------, above) demonstrated a response at three months. Although there is overlap between responders and non-responder in tumours expressing ER at lower levels, this could clearly be of clinical significance.

Figure 13.14 Pre-Treatment Oestrogen Receptor Levels as Determined by ER-ICA and Subsequent Overall Response to Tamoxifen.

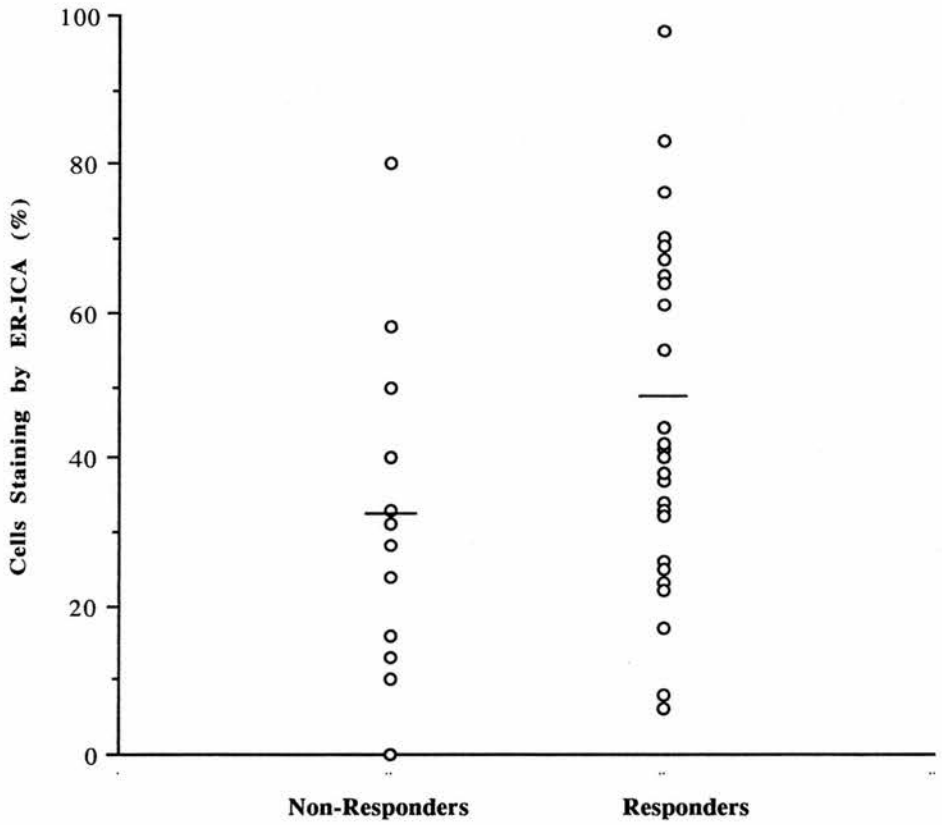


Figure 13.14 shows the relationship between pre-treatment ER levels as determined by ER-ICA on FNA samples and the subsequent overall response to tamoxifen. In this group of 50 tumours there is a significantly higher median number of cells staining by ER-ICA in subsequent responders compared to non-responders (Mann-Whitney U-statistic = 141.5, p = 0.0294). Unlike the data for ER assay by EIA there is a considerable degree of overlap in individual ER-ICA values in the responding and non-responding groups.

Figure 13.15 Pre-Treatment Oestrogen Receptor Levels as Determined by EIA and Subsequent Degree of Response to Tamoxifen.

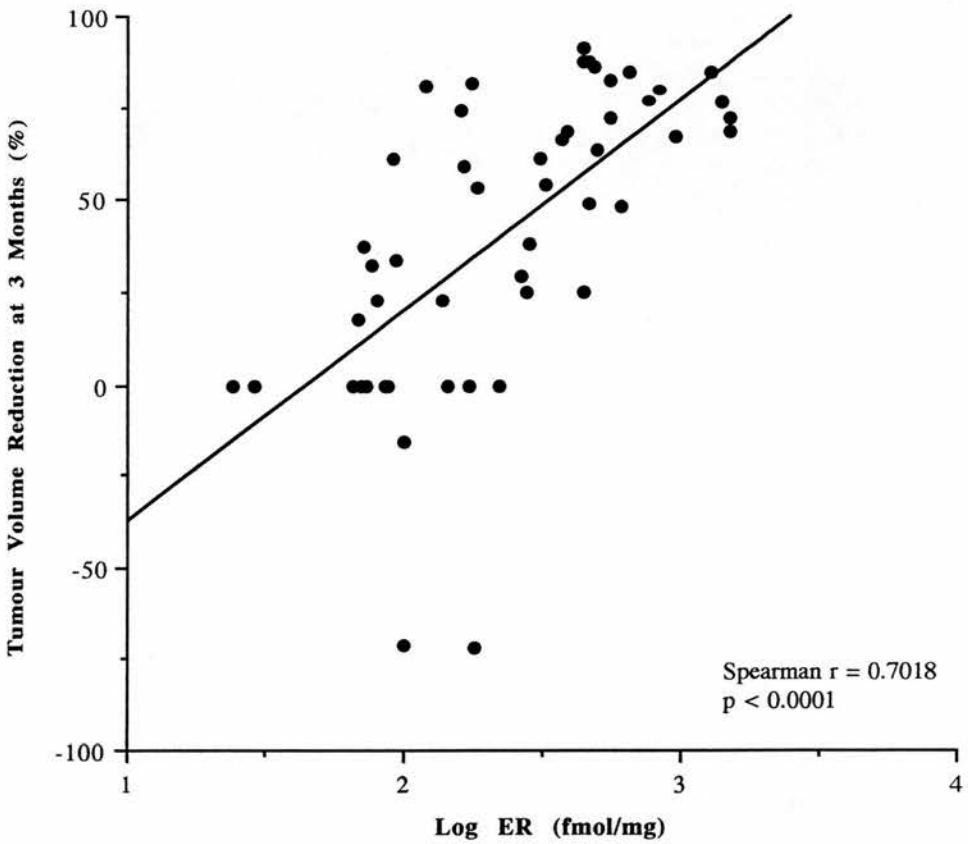


Figure 13.15 shows the relationship, in 50 tumours, between pre-treatment ER levels as determined by EIA and the degree of response as measured by the reduction in tumour volume after three months of therapy. There is a highly significant correlation.

Figure 13.16 Pre-Treatment Oestrogen Receptor Levels as Determined by ER-ICA and Subsequent Degree of Response to Tamoxifen.

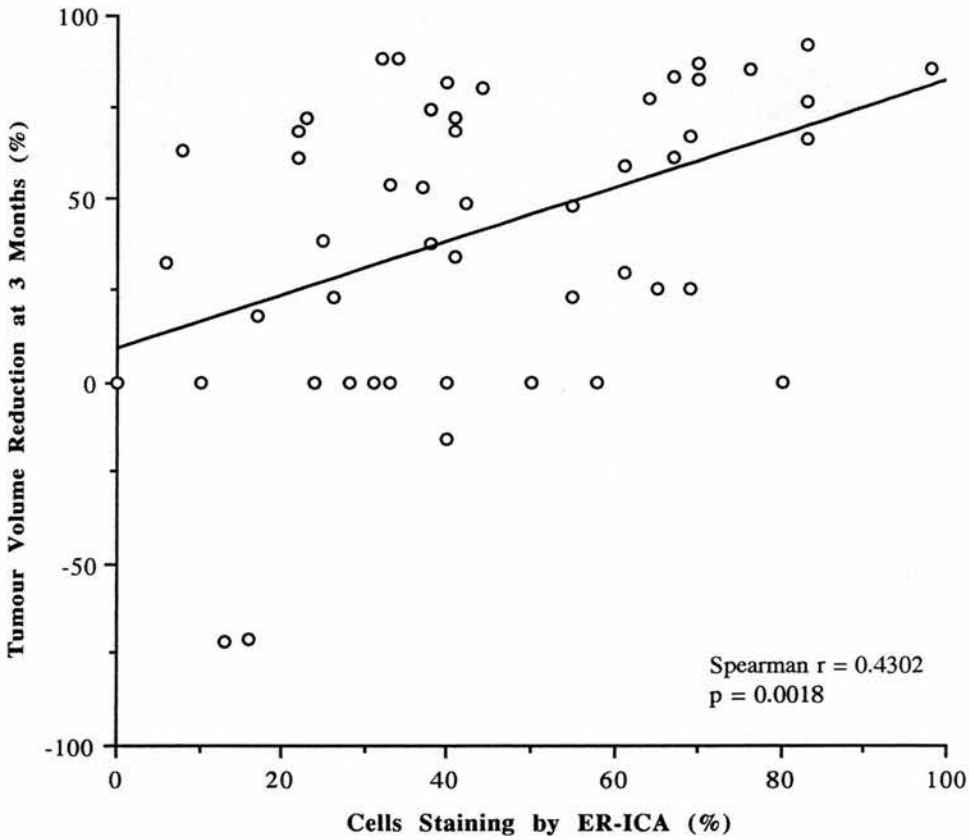


Figure 13.16 shows the relationship between pre-treatment ER expression, as determined by ER-ICA of cells obtained by FNA, and tumour response after three months of therapy. There is a significant correlation between these two parameters.

13.3 The Clinical Response to Chemotherapy and Hormone Manipulation in the Trial of Primary Systemic Therapy

Forty-six patients were recruited and randomised in the Edinburgh trial of primary systemic therapy. Twenty-two patients were randomised to receive primary therapy and 24 to undergo conventional treatment with primary surgery and standard adjuvant treatment as necessary. Their were two trial violations in the primary therapy arm; one patient decided to proceed to conventional therapy after randomisation, and one patient required repeat wedge biopsies to confirm the histological diagnosis with the result that there was little identifiable tumour left to monitor response. Both these patients underwent conventional therapy and are included in that arm for follow-up purposes. Five patients were treated by primary systemic therapy as per protocol outwith the trial through their personal choice. For the purposes of this thesis they will be included with the patients registered in the trial. This makes totals of 25 and 26 patients included in the primary systemic and conventional therapy arms of the trial respectively. The mean ages of patients assigned to the primary systemic and conventional therapy groups respectively were 51.7 years (range: 32-68) and 51.8 years (range: 27-68).

The clinical responses observed in those patients undergoing primary sytemic therapy are summarised in Table 13.3. Fifteen patients with ER poor tumours (<20 fmol/mg cytosol protein by EIA of wedge biopsy material) were given four pre-operative cycles of chemotherapy with cyclophosphamide, adriamycin and prednisolone (CAP). Fourteen patients responded with an average decrease in tumour volume of 87% (range: 58 to 100%) with three patients acheiving a complete pathological response. A plot of the variation in mean tumour volume over time is shown in Figure 13.17. One patient with a borderline ER positive tumour and who was pre-menopausal at presentation received three months treatment with the LHRH agonist, Goserelin. With no response to this therapy she received four cycles of CAP with a moderate response. Of the remaining nine patients with ER positive tumours seven responded to tamoxifen with a mean decrease in tumour volume of 70%. Individual plots illustrating the decrease in tumour volume over time are shown in Figure 13.18. The two patients who did not respond to tamoxifen also failed to respond when switched to CAP chemotherapy.

The numbers of patients studied are too small to make significant conclusions about patterns of response but given this limitation the CAP regime appears to have significant activity in terms of short-term response. A mean decrease in tumour volume of 87%, as seen in this series, would allow significant numbers of patients to avoid mastectomy. This option was not available in the Edinburgh protocol and so all

patients proceeded to mastectomy regardless of the degree of response. The avoidance of mastectomy has, however, been cited by other authors as an important principle behind the use of primary systemic therapy (see Chapter 2).

The prime aim of the Edinburgh study was to observe long-term survival in a group of patients randomised to either undergo primary systemic therapy or conventional primary surgery and adjuvant therapy. To date, in the series reported here, the mean follow-up times are 24.9 months (range: 15-31 months) and 24.9 months (range: 12-36 months) respectively. These are relatively short periods of time in the natural history of breast cancer and it is thus difficult to make significant comments on relapse-free and overall survival times in this group at present.

Table 13.3 The Response to Treatment in 25 Patients Treated According to the Protocol of the Edinburgh Trial of Primary Systemic Therapy

Patient No.	Treatment	Stage	Clin Diam	Ln Vol (USS)	4 Weeks	8 Weeks	12 Weeks
2	C.A.P.	T2N1M0	5.0	2.3	66.2	71.4	100.0
5	C.A.P.	T2N1M0	4.9	2.0	62.0	85.9	86.4
7	C.A.P.	T3N0M0	6.3	2.9	*	82.3	58.1
9	C.A.P.	T2N1M0	2.9	0.3	0.0	0.0	0.0
10	C.A.P.	T2N0M0	5.0	3.2	59.1	90.0	95.3
12	C.A.P.	T2N1M0	3.1	2.2	74.6	96.0	96.3
15	C.A.P.	T2N0M0	4.1	2.6	28.1	62.6	68.8
16	C.A.P.	T3N0M0	5.9	3.5	64.2	79.1	95.8
18	C.A.P.	T3N0M0	5.6	2.8	45.5	71.2	68.6
20	C.A.P.	T2N0M0	3.5	2.6	53.0	91.0	95.6
21	C.A.P.	T3N1M0	5.1	2.4	62.0	82.0	90.0
b	C.A.P.	T3N1M0	4.8	2.9	78.6	92.2	95.4
c	C.A.P.	T3N1M0	5.9	3.4	62.5	86.8	94.9
d	C.A.P.	T3N1M0	5.8	3.5	38.9	65.5	73.4
e	C.A.P.	T2N0M0	4.6	2.9	79.0	92.5	98.8
8	LHRH + C.A.P.	T3N1M0	6.2	3.4	0.0	0.0	0.0
11	TAM + C.A.P.	T2N0M0	2.9	1.5	0.0	0.0	5.4
4	TAM + C.A.P.	T3N1M0	8.2	3.6	0.0	0.0	0.0
1	TAM	T2N1M0	4.9	2.4	38.2	74.8	77.0
6	TAM	T2N0M0	4.0	2.1	57.5	74.4	73.3
13	TAM	T2N0M0	3.9	1.6	0.0	45.1	65.8
14	TAM	T2N0M0	3.8	1.9	31.2	48.0	40.3
19	TAM	T2N0M0	3.2	2.0	66.2	93.6	99.0
22	TAM	T2N0M0	3.8	1.7	0.0	23.1	40.0
a	TAM	T2N0M0	4.6	2.1	62.3	86.1	93.3

Table 13.3 shows the responses measured in 25 patients given either chemo- or hormonal therapy, or both, according to the protocol of the Edinburgh Trial of Primary Systemic Therapy.

C.A.P. = Cyclophosphamide, Adriamycin and Prednisolone chemotherapy.

LHRH = LHRH Agonist, Goserelin.

TAM = Tamoxifen.

Clin Diam = Tumour diameter measured by callipers (cm).

Response at 4, 8 and 12 weeks is expressed in terms of percentage reduction in tumour volume as assessed by ultrasound.

Figure 13.17 The Tumour Response over Time in 14 Patients Treated with Primary C.A.P. Chemotherapy.

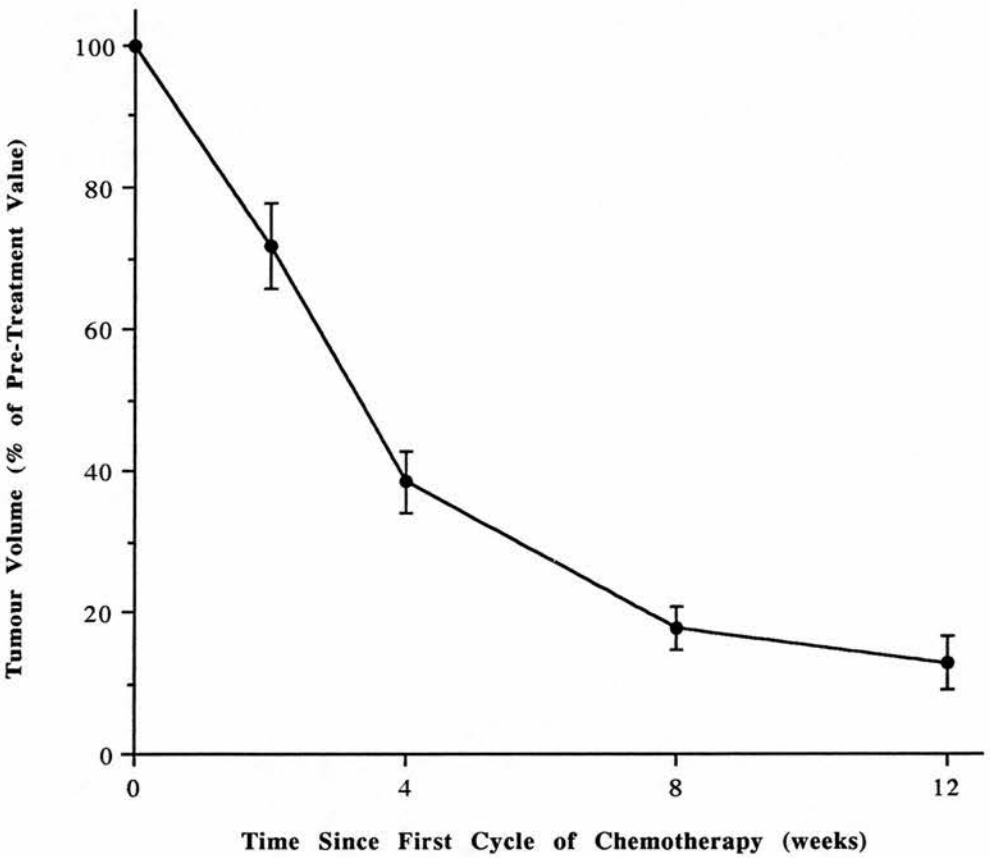


Figure 13.17 shows the response of 14 breast tumours treated with four, three-weekly, cycles of primary C.A.P. chemotherapy. The values plotted are the means of the percentage tumour volumes (as compared with pre-treatment volumes) at the times indicated. The volumes were all measured by ultrasound. The mean value at the two week time point represents data from only five of the 14 tumours but is included in an attempt to improve definition of the early response.

Figure 13.18 The Tumour Response over Time in 7 Patients Treated with Primary Tamoxifen Therapy

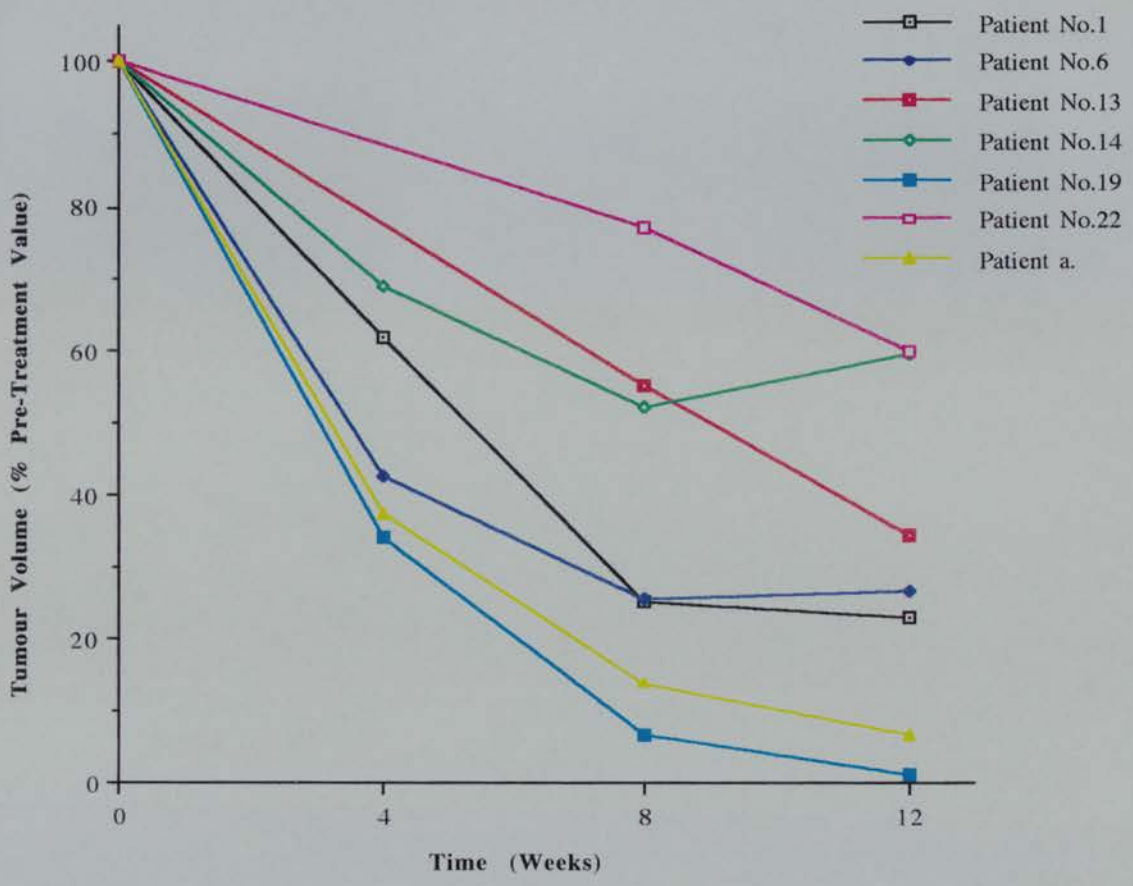


Figure 13.18 shows individual response curves for the seven tumours treated with tamoxifen as part of the Trial of Primary Systemic Therapy. Tumour volumes were assessed by serial ultrasound scans and related to the pre-treatment volume to measure response. Patient numbers refer to previous tables of clinical data presented for patients recruited to the trial.

Chapter 14: Biological Factors Predicting and Reflecting the Response to Primary Systemic Therapy

In Chapter 13 the clinical responses, as observed by serial measurements of individual tumours were reported in two groups of patients treated with either primary tamoxifen or chemotherapy. The data thus accumulated provided a basis on which to examine the corresponding cellular characteristics of the tumours studied. Tumour material was examined by both immunohistochemistry (reported below) and flow cytometry (Chapter 15).

14.1 Tumours from Patients Treated with Primary Tamoxifen Therapy

Fifty-two tumours were assessed by immunohistochemistry. Patients had been treated for a mean of 18.8 weeks with the response recorded over the first 12 weeks of threrapy. Material was available from these tumours from pre-treatment wedge biopsies and post-treatment definitive surgical procedures.

Tissue sections were stained with antibodies to seven different antigens; Ki-S1, Bcl-2 protein, pS2, c-*erb* B-2, *P* - Glycoprotein, EGF Receptor and p53. The relationship of these antigens to response was assessed both as predictive factors prior to treatment and as reflective factors by changes in expression with treatment. Oestrogen receptor levels have been shown in chapter 14 to be significantly related both to the overall and the degree of response to tamoxifen. EIA values for receptor content were available for all but one of the tumours prior to treatment and could thus be compared to the expression of these other antigens as a predictor of response.

A full table of immunohistochemical results and relevant clinical data is shown in Table 14.1. The results for individual markers will be presented initially and then the results of a multi-variate analysis will be discussed.

Table 14.1 Immunohistochemical Data from 52 Elderly Patients Before and After Primary Tamoxifen Therapy

Patient	Rx	Sample	Ki-S1	pS2	c-erbB-2	P-Glyc.	Bcl-2	EGFR	p53	ER (EIA)	L.N.	Response
1		Pre	2	3	0	0	1	0	0	606		69%
	19	Post	1	3	0	0	1	3	0	96	n/a	
2		Pre	2	0	0	0	2	2	0	23		73%
	21	Post	0	2	0	0	1	0	1	46	+	
3		Pre	2	0	*	2	0	0	0	325		54%
	18	Post	1	0	0	0	1	2	0	18	-	
4		Pre	1	1	0	1	3	1	0	29		Static
	22	Post	1	0	0	2	2	0	0	27	-	
5		Pre	0	0	0	0	1	0	0	86		Static
	18	Post	1	0	0	2	1	1	0	58	+	
6		Pre	1	2	0	0	1	0	0	605		48%
	16	Post	0	2	0	0	0	2	0	97	-	
7		Pre	2	2	1	0	3	3	0	444		88%
	15	Post	0	3	0	0	2	3	0	71	-	
8		Pre	1	0	0	1	1	2	0	*		34%
	21	Post	0	2	1	2	3	2	0	141	-	
9		Pre	2	2	0	3	0	1	0	87		Static
	18	Post	0	2	1	2	3	3	0	n/a	+	
10		Pre	1	2	0	0	2	0	0	445		91%
	15	Post	0	2	1	1	0	0	0	n/a	-	
11		Pre	2	0	2	0	3	1	1	79		23%
	19	Post	0	0	0	0	3	0	3	23	+	
12		Pre	0	0	0	0	1	0	0	170		Static
	18	Post	1	1	3	1	1	0	0	26	-	
13		Pre	1	0	2	0	0	0	0	68		18%
	20	Post	0	0	1	0	0	0	0	65	-	
14		Pre	1	1	0	1	2	0	0	290		33%
	25	Post	0	1	0	0	0	0	0	102	-	
15		Pre	0	0	1	0	3	0	0	70		Static
	27	Post	1	0	0	0	2	0	0	58	+	
16		Pre	0	0	3	0	3	0	0	384		68%
	19	Post	0	2	3	0	2	0	0	168	-	
17		Pre	2	3	3	0	1	0	0	498		63%
	12	Post	2	2	3	0	0	0	0	75	+	
18		Pre	1	3	0	1	1	0	1	186		53%
	18	Post	1	3	0	2	0	0	1	67	-	
19		Pre	2	3	0	0	3	0	0	510		45%
	19	Post	2	2	3	0	2	0	*	92	+	
20		Pre	0	0	0	1	0	0	0	222		Static

Table 14.1 Continued

Patient	Rx	Sample	Ki-S1	pS2	c-erbB-2	P-Glyc.	Bcl-2	EGFR	p53	ER (EIA)	L.N.	Response
	26	Post	1	0	0	3	2	0	0	90	+	
21		Pre	3	3	0	2	0	0	0	72		37%
	23	Post	0	2	0	0	0	1	0	n/a	+	
22		Pre	1	0	0	0	2	3	0	93		34%
	12	Post	1	0	0	0	1	2	*	0	-	
23		Pre	0	0	0	0	3	3	n/a	964		67%
	19	Post	0	0	0	0	*	0	0	n/a	-	
24		Pre	1	2	0	0	2	2	0	262		29%
	21	Post	2	3	1	0	2	1	0	n/a	-	
25		Pre	0	2	0	1	3	0	0	463		49%
	18	Post	0	2	0	1	1	1	0	3	+	
26		Pre	2	2	0	1	2	0	0	837		80%
	12	Post	3	1	0	1	1	0	0	n/a	+	
27		Pre	2	1	0	0	2	0	3	70		Static
	24	Post	3	1	2	0	0	0	3	48	-	
28		Pre	0	2	0	2	2	0	0	143		27%
	36	Post	0	0	0	1	3	0	n/a	n/a	+	
29		Pre	1	3	3	1	0	0	0	384		Static
	20	Post	0	0	0	0	3	2	0	n/a	+	
30		Pre	2	0	0	0	1	0	0	557		72%
	18	Post	0	1	0	0	1	0	0	128	+	
31		Pre	2	3	0	0	1	0	0	76		32%
	20	Post	1	1	1	0	1	0	0	116	-	
32		Pre	1	0	1	0	1	0	0	285		Static
	26	Post	2	0	0	0	1	0	0	56	+	
33		Pre	2	0	0	0	3	0	0	1000		85%
	14	Post	0	0	0	0	2	0	0	83	+	
34		Pre	1	0	0	2	2	0	0	177		82%
	14	Post	1	0	1	1	0	0	n/a	42	+	
35		Pre	1	0	3	0	1	0	0	65		Static
	28	Post	0	1	3	1	0	0	0	40	+	
36		Pre	1	1	0	2	1	0	0	166		59%
	17	Post	0	2	2	2	1	0	0	81	-	
37		Pre	1	0	0	0	1	0	1	292		38%
	15	Post	0	3	0	0	1	1	0	n/a	+	
38		Pre	0	1	0	0	3	0	0	1496		65%
	18	Post	0	3	0	0	0	0	*	n/a	+	
39		Pre	1	2	3	3	2	0	0	73		Static
	12	Post	2	1	0	2	1	0	0	12	-	

Table 14.1 Continued

Patient	Rx	Sample	Ki-S1	pS2	c-erbB-2	P-Glyc.	Bcl-2	EGFR	p53	ER (EIA)	L.N.	Response
40		Pre	2	0	1	2	1	0	0	279		25%
	18	Post	1	0	2	2	1	0	0	16	-	
41		Pre	0	0	0	1	3	0	0	1388		76%
	18	Post	0	1	0	0	3	0	0	n/a	-	
42		Pre	2	0	3	2	3	1	0	306		61%
	18	Post	0	0	0	0	0	0	0	60	-	
43		Pre	2	0	0	0	1	0	0	40		Progression
	19	Post	1	1	0	1	1	0	0	64	+	
44		Pre	2	1	0	1	1	0	*	201		Static
	17	Post	2	1	0	3	3	0	*	35	-	
45		Pre	1	1	0	0	1	1	0	134		Static
	18	Post	0	0	0	3	2	2	2	99	+	
46		Pre	1	0	1	0	2	0	0	120		81%
	25	Post	0	0	0	1	1	0	0	58	-	
47		Pre	0	2	0	0	2	1	*	16		Static
	30	Post	0	2	0	0	*	0	*	100	+	
48		Pre	1	0	0	1	3	0	0	620		40%
	12	Post	0	2	0	1	2	0	0	22	+	
49		Pre	1	2	0	0	0	0	0	552		77%
	12	Post	0	1	0	0	3	0	0	109	-	
50		Pre	0	0	1	1	1	0	0	25		66%
	12	Post	1	0	1	1	0	1	2	45	-	
51		Pre	0	1	0	1	3	0	0	623		73%
	12	Post	0	2	2	2	2	0	0	77	+	
52		Pre	1	0	0	0	3	0	0	386		99%
	12	Post	0	0	0	0	3	3	0	n/a	-	

Table 14.1 shows all the immunohistochemical data from tumour samples obtained pre and post tamoxifen treatment. Sections stained with each individual antibody were scored by an independent single pathologist. The scoring system used denotes the number of tumour cells stained; 0 = <5%, 1 = 5-25%, 2 = 25-75% and 3 = >75% of cells stained.

Rx is the treatment period in weeks between commencement of therapy and definitive surgery.

ER (EIA) is the Oestrogen receptor content measured in fmol/mg cytosol protein. There were several tumours with insufficient material in the post treatment specimen to obtain ER level.

L.N. is the axillary lymph node status determined from the axillary procedure performed at the time of definitive surgery and has been simply divided into nodal metastases present (+) or absent (-).

Response is measured by percentage decrease in tumour volume as assessed by ultrasound.

* denotes samples in which particular stains were unassessable despite repeated attempts.

14.2 The Expression of Ki-S1 and Response

Ki-S1 is a proliferation associated nuclear antigen which can be detected by a monoclonal-antibody both in fresh and formalin-fixed material. A typical example of a tumour strongly positive on staining with antibody to Ki-S1 is shown in Figure 14.1 In the group of 52 patients studied and reported in this section 37 (71%) demonstrated a response and 15 (29%) no response to treatment after three months. Prior to tamoxifen therapy, 39 (75%) patients had tumours with expression of Ki-S1 in greater than 5% of cells (Table 15.2a). On comparison of the degree of Ki-S1 staining (ie. the % of tumour cells stained) and subsequent overall response, there was no significant trend demonstrated. In the tissue obtained after treatment only 44% of tumours expressed detectable Ki-S1 but there was again no statistically significant trend in comparison to overall response (Table 14.2b). However when tabulating the change in expression and overall response a significant trend emerged with responding tumours demonstrating a decrease in the level of Ki-S1 expression and non-responders an increase in expression (Table 14.2c). The degree of the change in expression is tabulated with overall response in Table 14.2d, where the change in expression is represented by the change in staining index. A significant trend was once more demonstrated.

The relationship between Ki-S1 staining indices in the pre-treatment biopsy specimens and the degree of subsequent response as measured by ultrasound is shown in Figure 14.2. No significant correlation was apparent. The change in Ki-S1 staining index between tumour samples taken before and after treatment is related to reduction in tumour volume in Figures 14.3. There was a trend, which just reached statistical significance, for those tumours with the greatest decrease in volume to show the greatest decrease in Ki-S1 staining index.

Ki-S1 expression, therefore, in those patients studied, does not appear to predict for the overall or actual degree of response to tamoxifen. However the change in expression does reflect the degree of clinical response.

Figure 14.1 Immunohistochemical Staining for Ki-S1

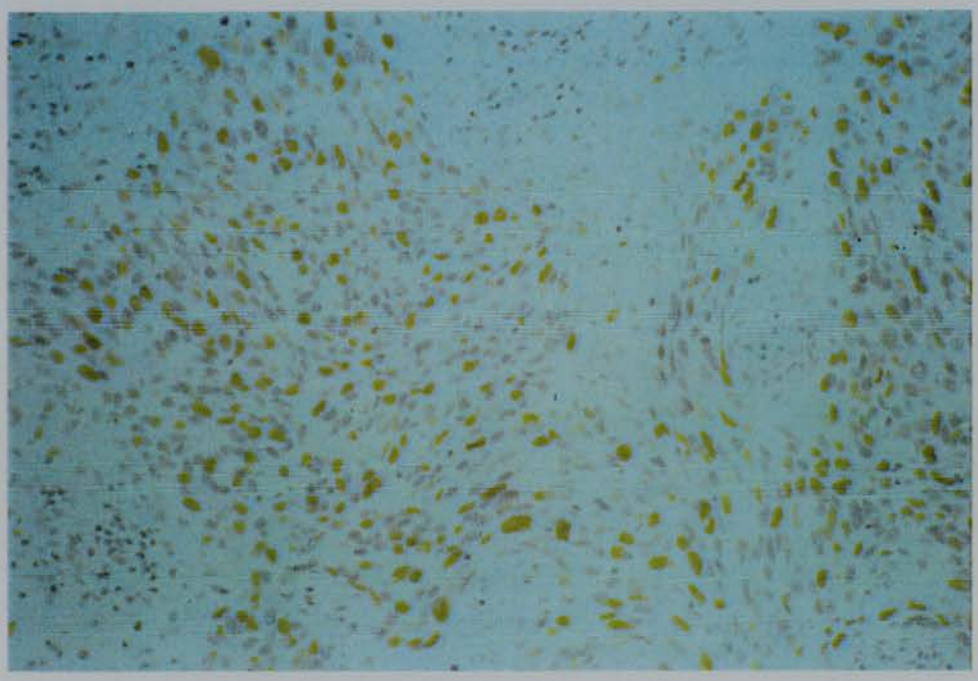


Figure 14.1 shows immunohistochemical staining with Ki-S1 monoclonal antibody in a section from a primary breast cancer prior to tamoxifen therapy (x40). Note the nuclear staining.

Table 14.2 The Expression of Ki-S1 before and after Tamoxifen Treatment

a) Before

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders(37)	8	15	13	1
Non-Responders(15)	5	6	4	0

X^2 Trend = 1.09

p = NS

b) After

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders(37)	24	8	4	1
Non-Responders(15)	5	6	3	1

X^2 Trend = 3.48

p = 0.06

c) Overall Change in Expression

	Change in No. of Cells Staining		
	Increase	No Change	Decrease
Responders (37)	3	11	23
Non-Responders (15)	7	3	5

X^2 Trend = 8.73

p =0.003

d) Degree of Change in Expression

	Change in Staining Index				
	-3	-2	-1	0	+1
Responders(37)	1	6	15	12	3
Non-Responders(15)	0	1	4	3	7

X^2 Trend = 6.42

p = 0.01

Table 14.2 shows immunohistochemical data from tumour tissue obtained before and after treatment with tamoxifen. Tumours are divided into responders and non-responders depending on the response obtained over three months of treatment as assessed by ultrasound scanning. Table a) demonstrates positive Ki-S1 staining (>5% of cells staining) in 39 of the 52 tumours (75%) prior to treatment. There is no significant relationship between the percentage of tumour cells staining and subsequent overall response. Post-treatment (Table b)) 23 tumours (44%) remined Ki-S1 positive but again there was no significant relationship between degree of staining and prior overall response. The inferred change in staining with treatment is demonstrated in Table c). There is a significant trend towards a decrease in staining in tumours which have demonstrated a response to tamoxifen. The degree of change is tabulated against overall clinical response in table d). The degree of change represents the difference between the pre- and post-treatment staining indices (where a staining index of <5% = 0, 5-25% = 1, 25-75% = 2 and >75% = 3), negative values representing a decrease.

Figure 14.2 Ki-S1 Staining Prior to Tamoxifen Treatment and Subsequent Clinical Response.

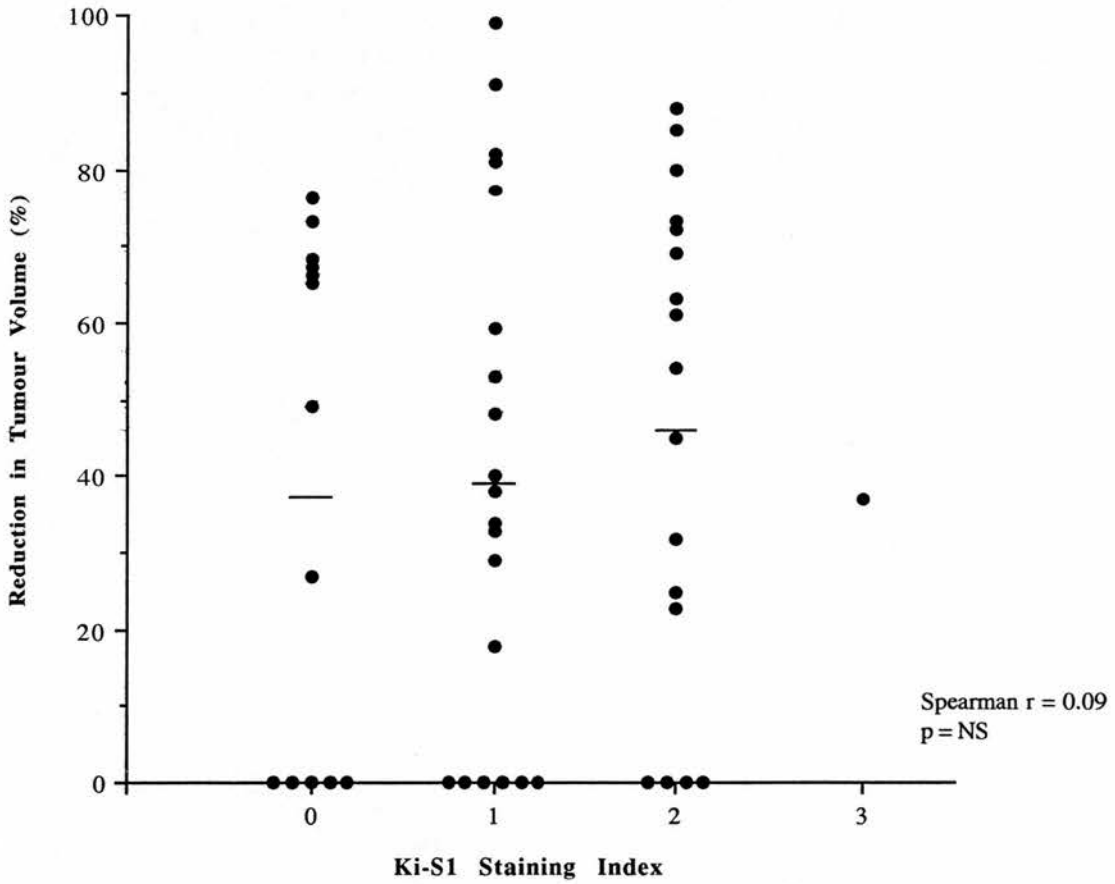


Figure 14.2 shows the relationship between Ki-S1 staining index prior to tamoxifen therapy and subsequent % reduction in tumour volume (as assessed by ultrasound). There is no significant difference between the median response of tumours with each of the staining indices.

Figure 14.3 Change in Ki-S1 Staining Index and Clinical Response Tamoxifen Treatment.

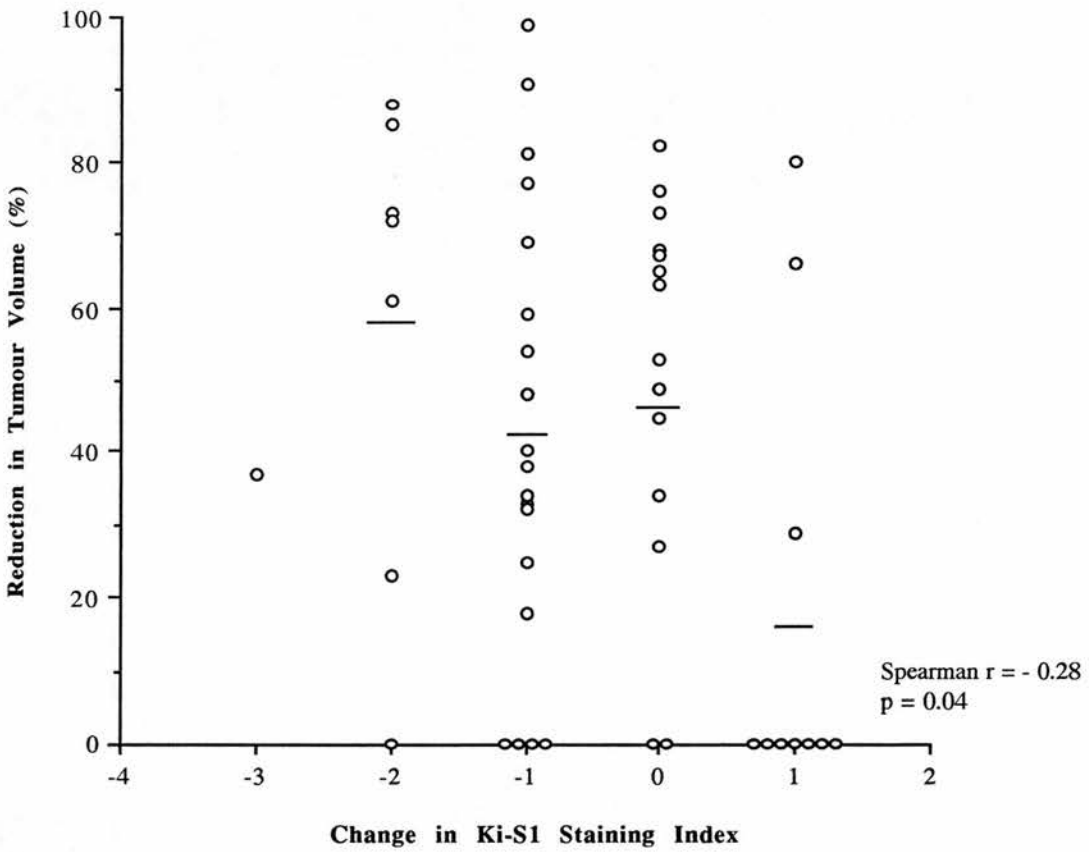


Figure 14.3 shows the relationship between the change in Ki-S1 staining indices with tamoxifen treatment and the % tumour volume reduction. There is a correlation, which just achieves statistical significance, such that greater tumour responses are associated with greater reductions in Ki-S1 staining indices.

14.3 The Expression of Bcl-2 and Response

The Bcl-2 protein is associated with the cellular mechanisms responsible for the resistance to death by apoptosis. Immunohistochemical staining for the protein was possible in 51 patients only. Despite repeated attempts tumour sections from one of the patients failed to survive the staining process. Of these 51 tumours, 44 (86%) were classed as staining positive for Bcl-2 prior to tamoxifen therapy and an example of positive staining is shown in Figure 14.4.

The relationships between Bcl-2 staining both before and after tamoxifen treatment and the overall clinical response are shown in Tables 14.3 a,b,c, and d.There was no significant trend in the degree of staining in tumour samples taken prior to treatment and the subsequent overall response (Table 14.3a). However, there was a significant relationship with the degree of response as measured by decrease in tumour volume (Figure 14.5), higher staining indices being associated with tumours displaying the greater reduction in volume. After treatment 37 (73%) tumours were positive for Bcl-2 staining and again no significant association was apparent between the degree of staining and overall response (Table 14.3b). There were, however, significant associations between the change in staining index and both the overall response (Table 14.3c and d) and the degree of response in terms of reduction in tumour volume (Figure 14.6). Decreased Bcl-2 staining was related to both qualitative and quantitative response.

The expression of Bcl-2 has previously been shown to be related to ER status and in this present series of tumours the quantitative relationship between these two parameters was examined. This relationship is illustrated graphically in Figure 14.7 and shows a positive correlation between Bcl-2 staining index and cellular levels of ER. In particular, those tumours with the highest staining index (grade 3) tended to have very high levels of ER.

Figure 14.4 Immunohistochemical Staining for Bcl-2

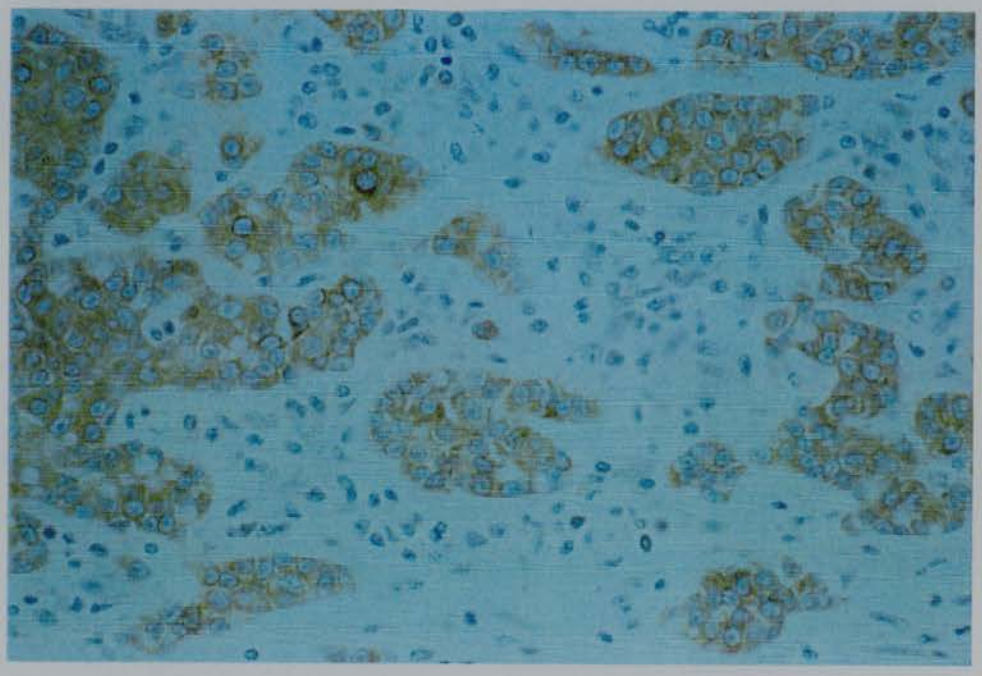


Figure 14.4 shows immunohistochemical staining with monoclonal antibody 124 to Bcl-2 protein in a section from a primary breast cancer prior to tamoxifen therapy (x40). Note the granular cytoplasmic staining.

Table 14.3 The Expression of Bcl-2 before and after Tamoxifen Treatment

a) Before

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders(37)	4	11	9	13
Non-Responders(14)	3	7	2	2

X^2 Trend = 3.27

p = NS

b) After

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders(37)	12	12	7	6
Non-Responders(14)	2	5	4	3

X^2 Trend = 1.34

p = NS

c) Overall Change in Expression

	Change in No. of Cells Staining		
	Increase	**No Change**	**Decrease**
Responders (37)	**4**	**12**	**21**
Non-Responders (14)	**5**	**4**	**5**

X^2 Trend = 3.73

p = 0.05

d) Degree of Change in Expression

	Change in Staining Index						
	-3	**-2**	**-1**	**0**	**+1**	**+2**	**+3**
Responders(37)	**3**	**4**	**14**	**12**	**2**	**1**	**1**
Non-Responders(14)	**0**	**1**	**4**	**4**	**1**	**2**	**2**

X^2 Trend = 5.24

p = 0.02

Table 14.3 shows the relationship of immunohistichemical staining of 51 breast tumours with Bcl-2 monoclonal antibody and overall response to 3 months of tamoxifen therapy. Tables 14.3a and b demonstrate that there is no significant trend in Bcl-2 expression before or after treatment when responding and non-responding patients are compared. Although the trend in overall change in staining with response does not quite reach significance, there is a significant trend when the degree of change in staining is compared with overall response (Tables 14.3c and d). Those tumours which show the greatest decrease in staining tend to be those which demonstrate reduction in volume with tamoxifen.

Figure 14.5 Bcl-2 Staining Prior to Tamoxifen Treatment and Subsequent Clinical Response.

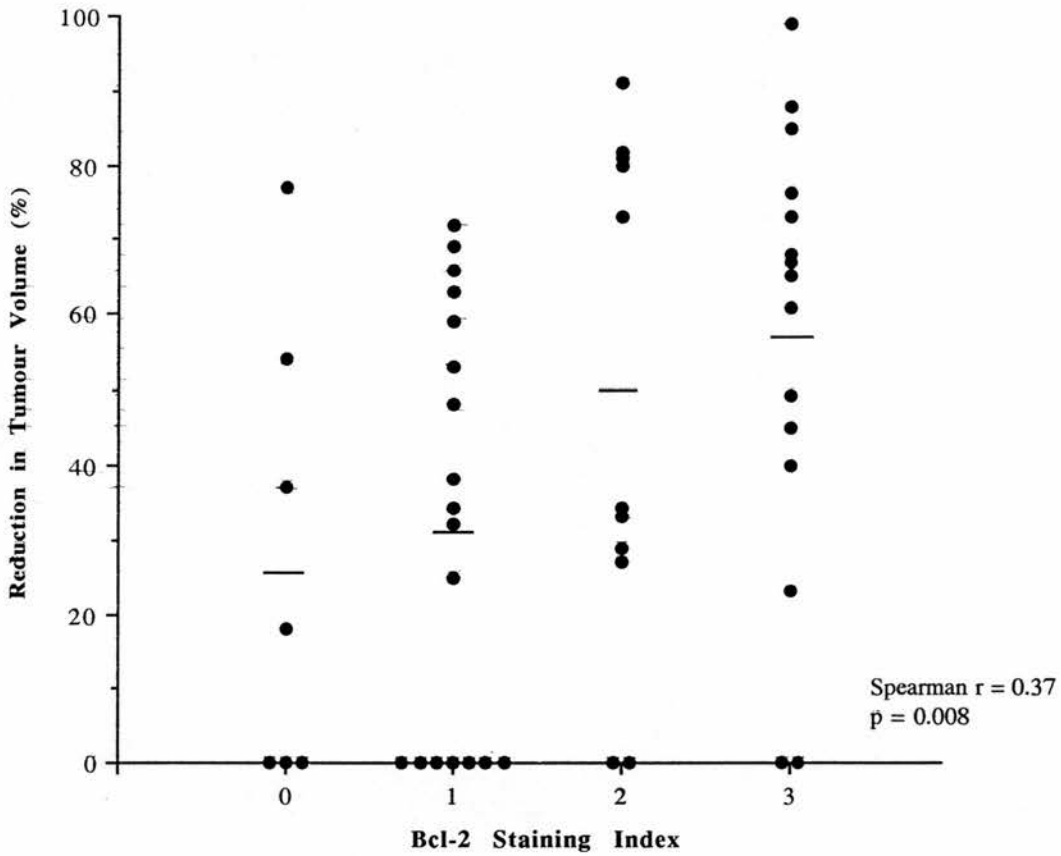


Figure 14.5 shows the relationship between Bcl-2 staining index in tumour tissue taken prior to treatment and tumour volume reduction with subsequent tamoxifen therapy. There is a significant correlation although there is a wide variation of responses for individual staining indices.

Figure 14.6 Change in Bcl-2 Staining Index and Clinical Response Tamoxifen Treatment.

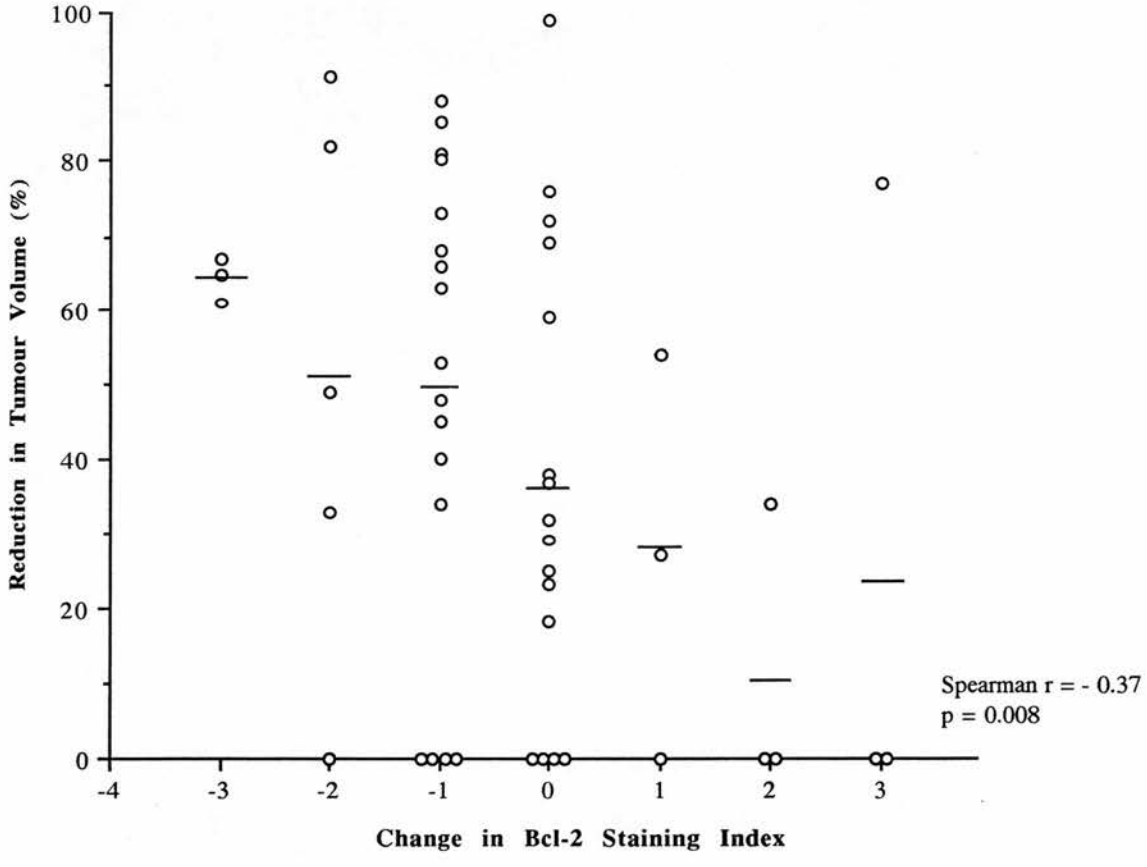


Figure 14.6 shows the relationship between the change in the Bcl-2 staining index and the reduction in tumour volume with tamoxifen therapy. There is a significant correlation in that those tumours showing the greater decreases in staining are those responding with greater reductions in tumour volume and those which show an increase in staining have a much smaller mean reduction in volume.

Figure 14.7 Bcl-2 Staining Index and Oestrogen Receptor Expression Prior to Tamoxifen Treatment

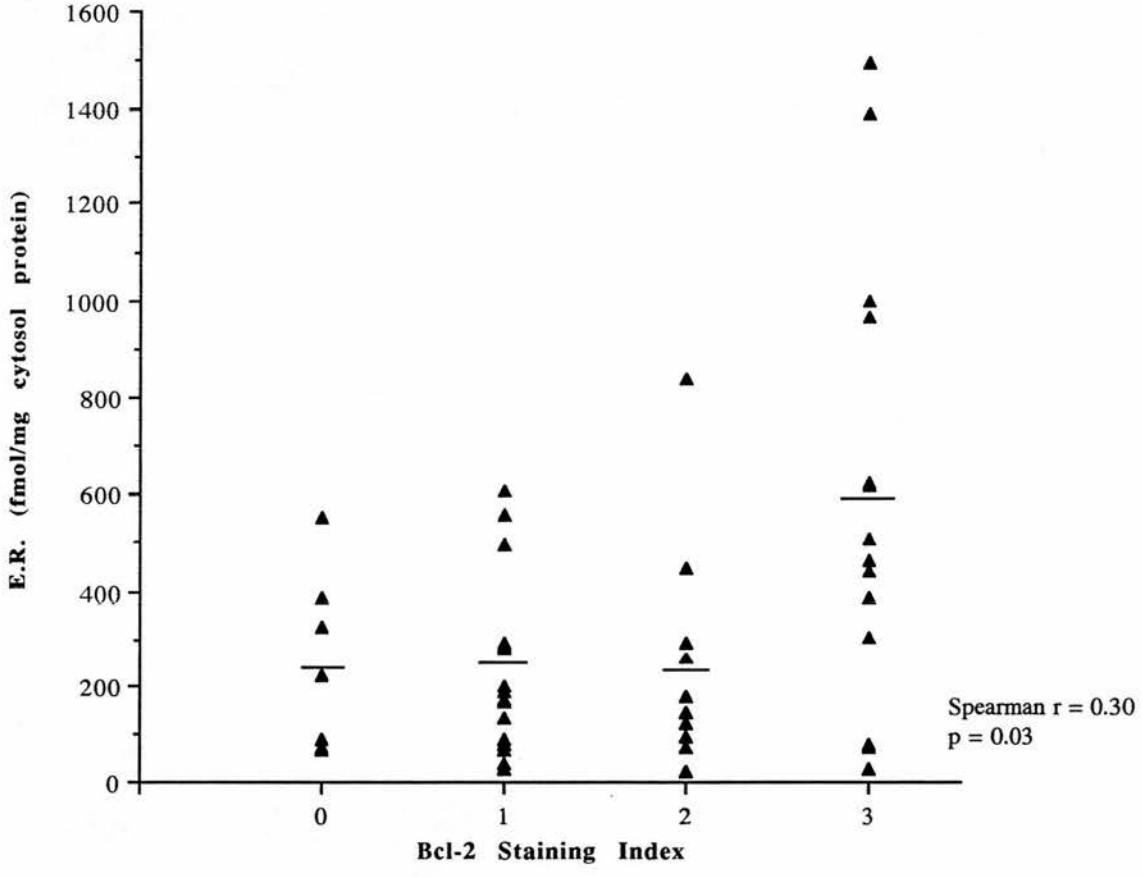


Figure 14.7 shows the relationship, prior to treatment, between Bcl-2 staining indices and ER expression as measured by EIA. There is a significant overall correlation but a wide range of values for ER at individual Bcl-2 staining indices. The median ER level associated with a Bcl-2 staining index of 3 is significantly higher than the levels associated with lower indices (e.g index 3 vs 2 Mann-Whitney U-statistic = 42.5, p = 0.04). There is no significant difference between median ER levels at Bcl-2 staining indices of 0 to 2.

14.4 The Interaction Between Ki-S1 and Bcl-2

Since tumour growth has been simplistically described in terms of the respective rates of cell proliferation and cell death, reduction in tumour volume could also be linked to these two processes. Associations between tumour response, the expression of a proliferation marker, such as Ki-S1 and the indirect marker for cell survival, Bcl-2 might, therefore, be expected. The expression of Bcl-2 is not known to be cell cycle dependent and therefore changes in expression would not be expected simply to reflect change in proliferation.

Although there were similar trends in the expression of both markers towards a decrease in responding tumours, the changes of each marker in individual tumours were largely independent, with only 11 of the 51 tumours showing concordant changes of expression (10 decreased, 1 increased), as summarised in Table 14.4a. Because of this independence, it was of interest to combine the changes in expression of the two markers. Thus Table 14.4b shows tumours categorised as displaying a net increase (an increase in both markers or an increase in one and no change in the other), a net decrease (the reverse changes) or a net no change (an increase in one marker and a decrease in the other or no change in either) in marker staining indices. These results show that the pattern of change in responding tumours is highly significantly different from that of non-responding tumours (and greater than when the markers had been considered separately). Furthermore, when the quantitative changes in Ki-S1 and Bcl-2 expression in individual tumours were added together, highly significant associations were obtained with both the overall response (Table 14.5) and the degree of response (Figure 14.8). Thus the use of a combination of changes in both markers results in a better correlation with response than that produced by either marker alone. In other words response can be associated with a decrease in either Ki-S1 (a decrease in proliferation) or Bcl-2 (an increase in cell death) or both and the best responses are seen in those tumours in which both markers decrease significantly.

Table 14.4 Combined Changes in Ki-S1 and Bcl-2 and Overall Response to Tamoxifen

a)

	Change in Staining Index		
	Both Increase	**Both Decrease**	**Others**
Responders (37)	**0**	**9**	**28**
Non-Responders (14)	**1**	**1**	**12**

b)

	Change in Staining Indices		
	Net Increase	**Net No Change**	**Net Decrease**
Responders (37)	**1**	**7**	**29**
Non-Responders (14)	**6**	**5**	**3**

X^2 Trend = 18.26

p = <0.0001

Table 14.4 shows the relationships between overall response and the combined changes in Ki-S1 and Bcl-2 staining indices. Table a) demonstrates the concordance of the changes in expression of each marker and table b) the overall change after summation of the changes in individual markers.

Table 14.5 The Sum of Changes in Ki-S1 and Bcl-2 Staining Indices and Response

	Sum of Changes in Staining Indices							
	-5	-3	-2	-1	0	+1	+2	+3
Responders(37)	1	8	7	13	4	3	1	0
Non-Responders(14)	0	0	1	3	3	4	2	2

X^2 Trend = 12.69

p = 0.0004

Table 14.5 shows the relationship between the sum of the changes in KI-S1 and Bcl-2 staining indices in individual tumours and overall response to tamoxifen. There is a highly significant trend, the greater decreases in the combined sum tending to be in responding tumours. The trend is more significant than with either marker alone.

Figure 14.8 The Sum of the Change in both Ki-S1 and Bcl-2 Staining Indices and Response to Tamoxifen

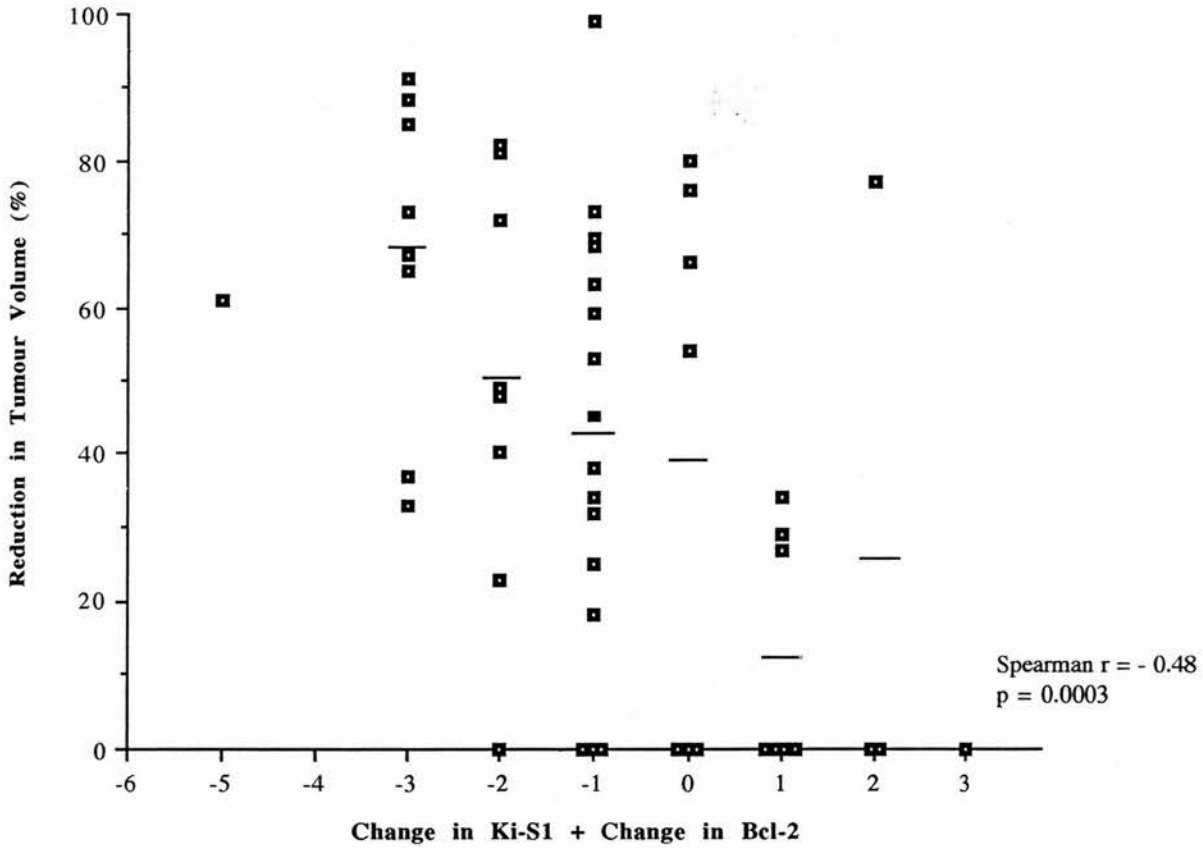


Figure 14.8 shows the relationship between tumour response, as measured by reduction in volume, and the sum of the changes in Ki-S1 and Bcl-2 staining indices in individual tumours. There is a highly significant correlation with the greater total decreases in staining indices reflecting the greater reductions in tumour volume.

14.5 The Expression of pS2

The protein product of the oestrogen-inducible gene, pS2, was examined by means of immunohistochemistry in the study population of 52 patients receiving primary therapy with tamoxifen. Staining for pS2 was detectable in >5% of tumour cells in 26 (50%) patients prior to therapy and a typical example of positive staining is shown in Figure 14.9. There was no significant association between initial staining index and tumours subsequently responding or not responding to treatment (Table 14.6a). Expression of pS2, therefore, did not predict for overall response in this series and in addition did not correlate with the degree of response in terms of reduction in tumour volume (Figure 14.13).

The pS2 gene appears to be inducible by oestrogens in certain systems and it might, therefore, be that a relationship exists with the ER content of individual tumours. No such association was apparent in staining of the pre-treatment samples (Figure 14.10). However there was a statistically significant relationship between ER prior to therapy and the pS2 staining index after treatment (Figure 14.11). This may reflect the ability of tamoxifen to act as a partial agonist at the oestrogen receptor and induce pS2. Despite this association, the correlation between pre-treatment ER and the change in pS2 staining index with treatment was not significant (Figure 14.12). Additionally, there were significantly higher levels of pS2 staining in the post-treatment samples from responding tumours when compared to those which had not responded to tamoxifen (Table 14.6b) . However, when considering the association of overall change in pS2 staining with response, although there was a trend towards a greater number of the responding tumours showing increase in pS2 expression this did not reach statistical significance (Table 14.6c) The degree of change in staining index also did not significantly relate to either the overall (Table 14.6d) or the degree of response (Spearman $r = 0.1867$, $p = NS$, data not shown). These relationships held true when the subset of tumours which did not express pS2 either before or after therapy were removed from the analyses.

Figure 14.9 Immunohistochemical Staining for pS2

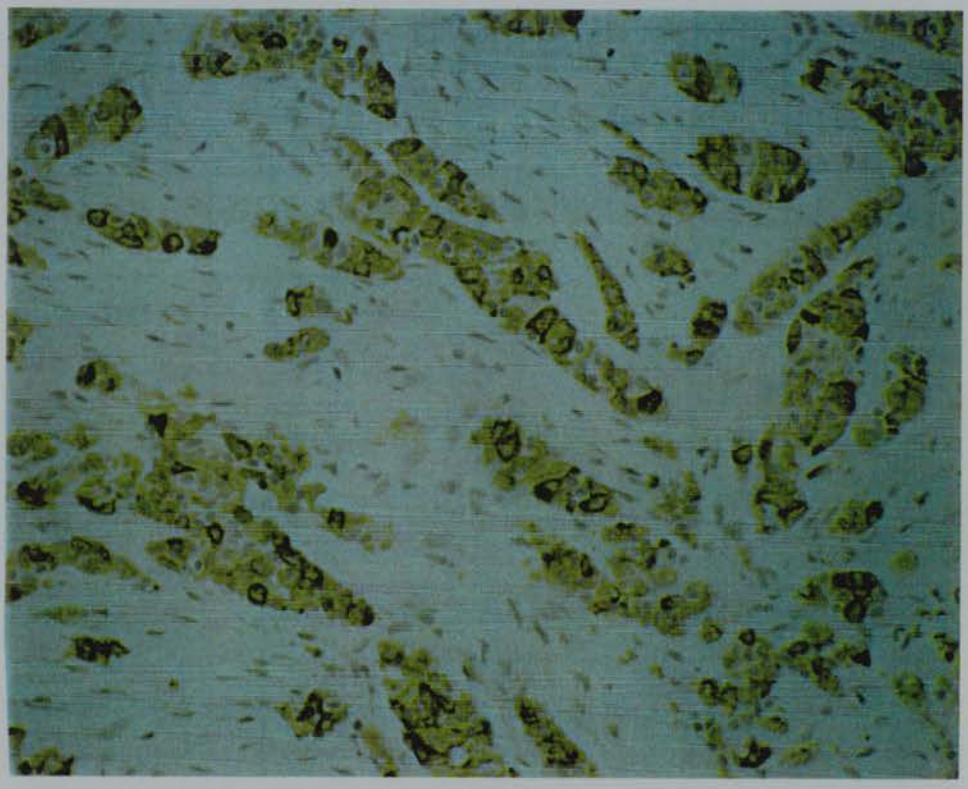


Figure 14.9 shows immunohistochemical staining with monoclonal antibody to pS2 protein in a section from a primary breast cancer prior to tamoxifen therapy (x40). Note the strong cytoplasmic staining.

Table 14.6 The Expression of pS2 Protein and Response to Tamoxifen

a) Before

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders(37)	19	4	8	6
Non-Responders(15)	7	4	3	1

X^2 Trend = 0.22

p = NS

b) After

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders(37)	13	6	12	6
Non-Responders(15)	7	6	2	0

X^2 Trend = 3.85

p = 0.05

c) Overall Change in Expression

	Change in No. of Cells Staining		
	Increase	No Change	Decrease
Responders(37)	12	18	7
Non-Responders(15)	3	8	4

X^2 Trend = 0.88

p = NS

c) Degree of Change in Expression

	Change in Staining Index						
	-3	-2	-1	0	+1	+2	+3
Responders(37)	0	2	5	18	6	5	1
Non-Responders(15)	1	0	3	8	3	0	0

X^2 Trend = 1.98

p = NS

Table 14.6 displays the immunohistochemical results for pS2 antibody staining of tumour sections from the fifty-two patients in the study population. Tables a) and b) show the degree of pS2 staining in the respondin and non responding tumours before and after treatment. There is a significant trend towards a greater proportion of cells staining in responding patients in the samples taken after therapy. Although a tendency for an increase in pS2 staining in responding patients is seen in tables c) and d) this does not reach statistical significance.

Figure 14.10 The Relationship Between the Expression of pS2 Protein and ER Content Prior to Tamoxifen

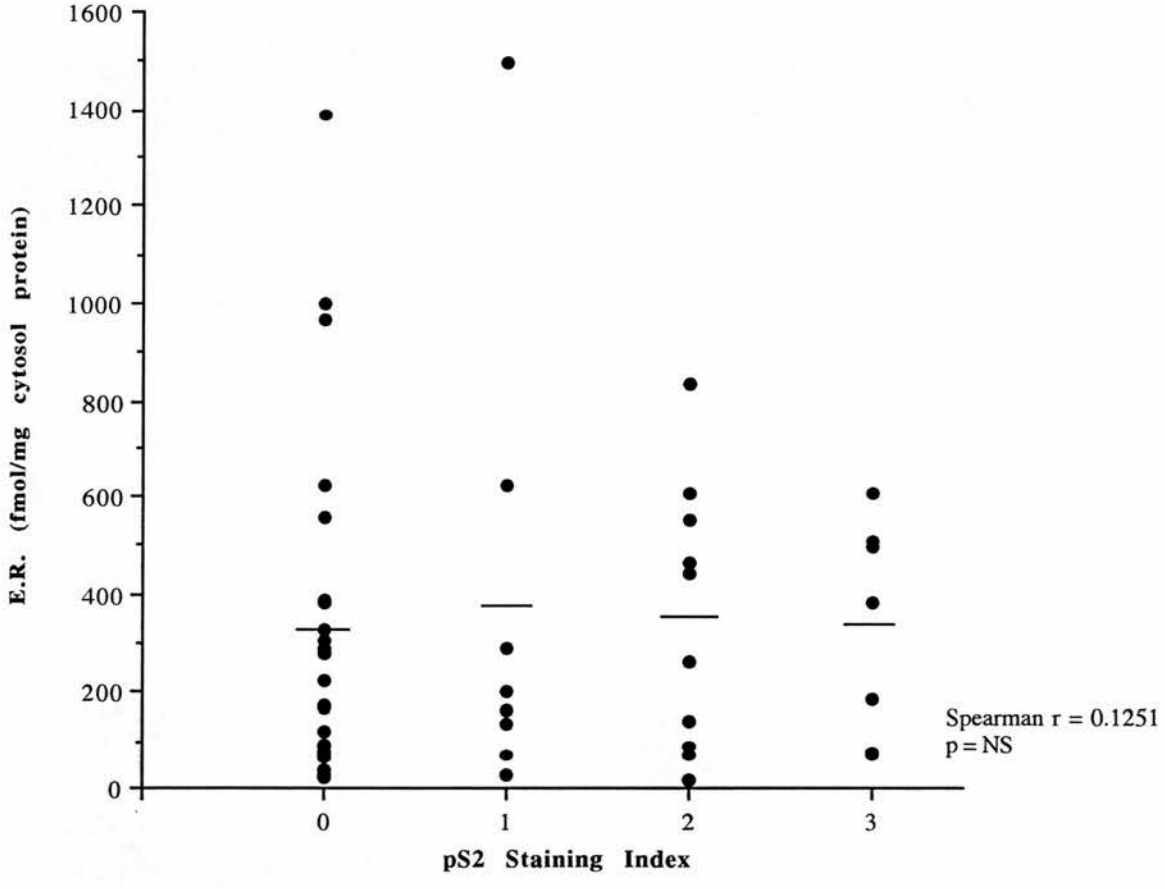


Figure 14.10 shows the relationship between pS2 staining index and ER content, as measured by EIA, in tissue taken from 51 tumours prior to tamoxifen treatment. One tumour from the study population of 52 could not be assessed for ER content. In this series there is no significant correlation between pS2 staining index and ER content.

Figure 14.11 The Relationship Between the Expression of pS2 Protein after Tamoxifen and ER Content Prior to Treatment

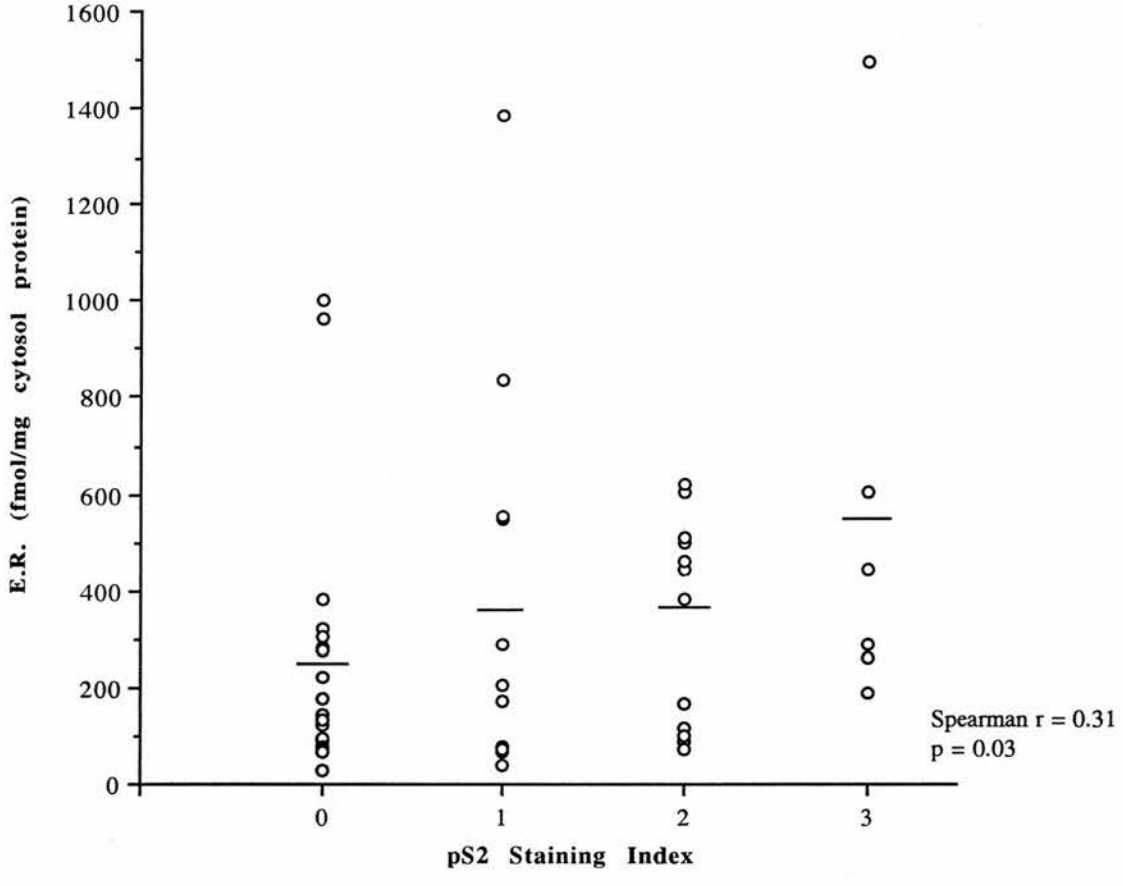


Figure 14.11 shows the relationship between pS2 staining index in tissue taken after treatment and the ER content of the same tumour prior to therapy. There is a statistically significant correlation between the two parameters with higher post-treatment staining levels associated with higher pre-treatment ER content.

Figure 14.12 The Pre-Treatment ER Content and the Change in pS2 Expression

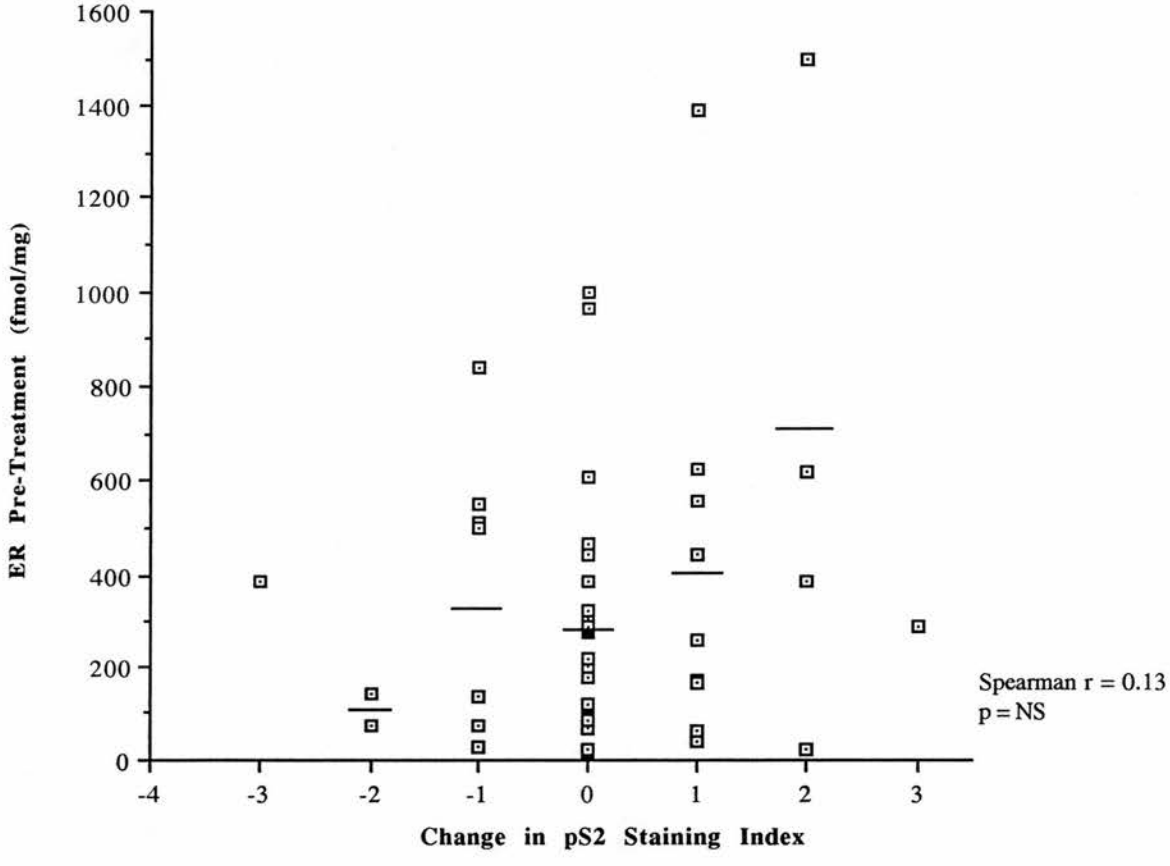


Figure 14.12 shows the relationship between the tumour ER content prior to treatment and the change in pS2 expresion with subsequent tamoxifen therapy. There is no significant correlation between these two parameters although there appears to be a trend towards a positive change in staining in tumours with higher levels of ER.

Figure 14.13 The Expression of pS2 and the Subsequent Response to Tamoxifen

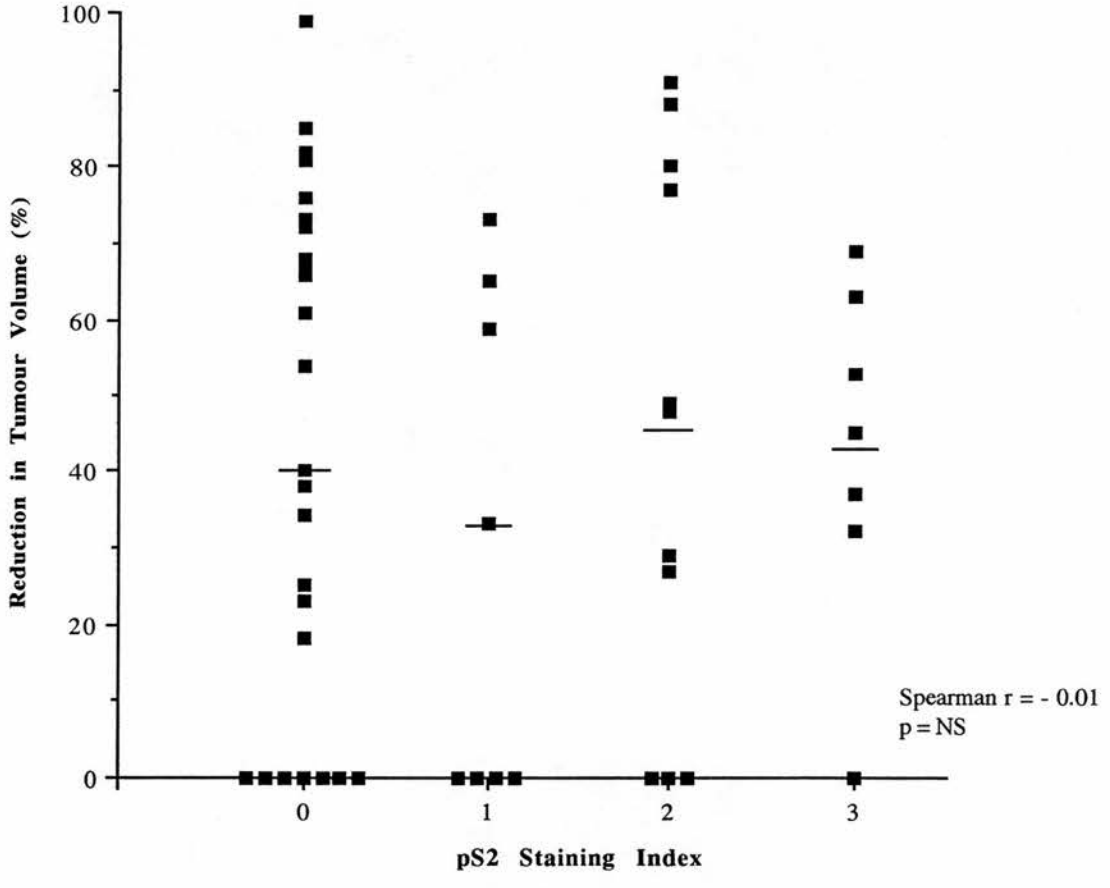


Figure 14.13 shows the relationship between pS2 expression prior to treatment and the subsequent response to tamoxifen as represented by reduction in tumour volume. There is no significant correlation between these parameters in this series of 52 patients. Prior to therapy 26 tumours displayed pS2 staining and in this subpopulation there was no significant correlation beween the degree of staining and response (Spearman r = 0.16, p = NS)

14.6 The Expression of c-*erb*B-2

Similar studies were performed in the series of tumours described to determine the relationship between the expression of c-*erb*B-2 and the clinical response to tamoxifen. An example of positive staining obtained with monoclonal antibody to the c-*erb*B-2 antigen is shown in Figure 14.14. As shown in Table 14.7a 14 (27%) of the 51 tumours demonstrated positive staining for c-*erb*B-2 prior to therapy. Futhermore, there was no significant difference in the degree of staining between tumours subsequently responding or non-responding to tamoxifen. Additionally there was no significant relationship between the staining index and the degree of response to treatment (Figure 14.15). After treatment 17 (33%) stained positive and again there was no significant difference in the degree of staining between responders and non-responders (Table 14.7b). A tendancy was apparent for an increase in staining in responding tumours with treatment (Tables 14.7c and d) but given the relatively small numbers of positively staining tumours this did not reach statistical significance. No significant relationship was detected between the change in c-*erb*B-2 staining index and the degree of the clinical response (Figure 14.16).

Figure 14.14 Immunohistochemical Staining for c-*erb*B-2

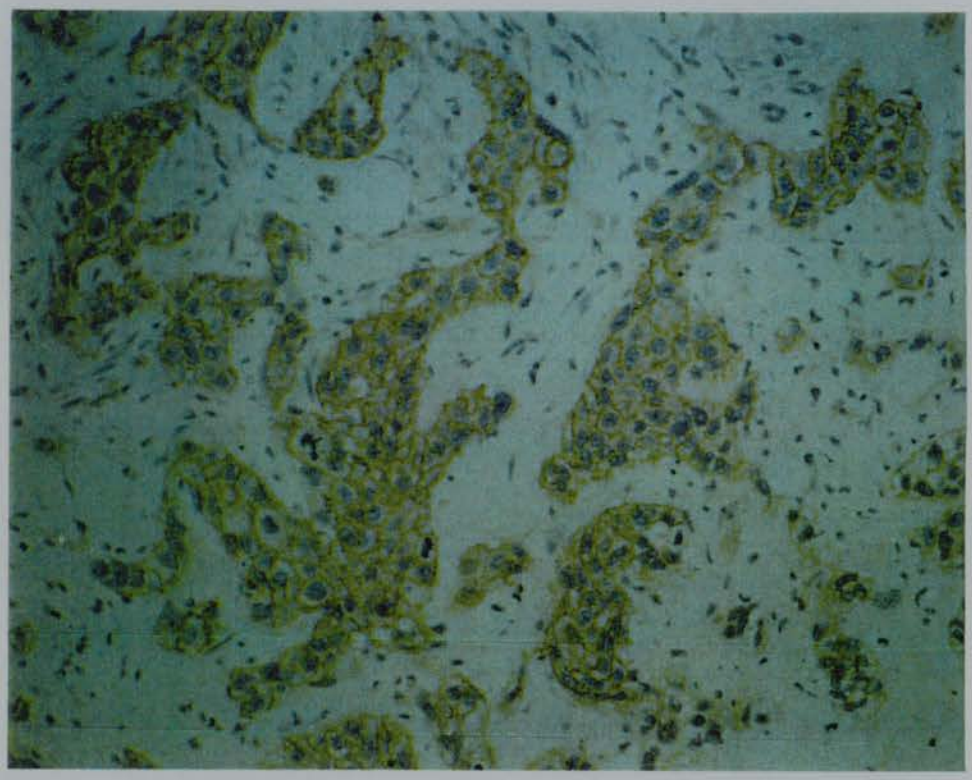


Figure 14.14 shows immunohistochemical staining with monoclonal antibody to c-*erb*B-2 protein in a section from a primary breast cancer prior to tamoxifen therapy (x40). Note the strong membrane staining.

Table 14.7 The Expression of c-*erb*B-2 Protein and Response to Tamoxifen

a) Before

	Cells Staining			
	<5%	**5-25%**	**25-75%**	**>75%**
Responders (36)	**27**	**4**	**2**	**3**
Non-Responders (15)	**10**	**2**	**0**	**3**

X^2 Trend = 0.6996

p = NS

b) After

	Cells Staining			
	<5%	**5-25%**	**25-75%**	**>75%**
Responders (36)	**23**	**7**	**3**	**3**
Non-Responders (15)	**11**	**1**	**1**	**2**

X^2 Trend = 0.001

p = NS

c) Change in Expression

	Change in No. of Cells Staining		
	Increase	**No Change**	**Decrease**
Responders(36)	**9**	**22**	**5**
Non-Responders(15)	**3**	**8**	**4**

X^2 Trend = 0.82

p = NS

d) Degree of Change in Expression

	Change in Staining Index						
	-3	**-2**	**-1**	**0**	**+1**	**+2**	**+3**
Responders(36)	**1**	**1**	**3**	**22**	**6**	**2**	**1**
Non-Responders(15)	**2**	**0**	**2**	**8**	**1**	**1**	**1**

X^2 Trend = 0.55

p = NS

Table 14.7 shows immunohistochemical data representing staining for c-*erb*B-2 in 51 tumours a) before and b) after treatment. There are no significant differences between the degrees of staining prior to and after treatment and the observed overall response. In addition there are no significant changes in c) the overall, and d) the degree of change in staining and clinical response.

Figure 14.15 The Expression of c-*erb*B-2 and the Response to Tamoxifen

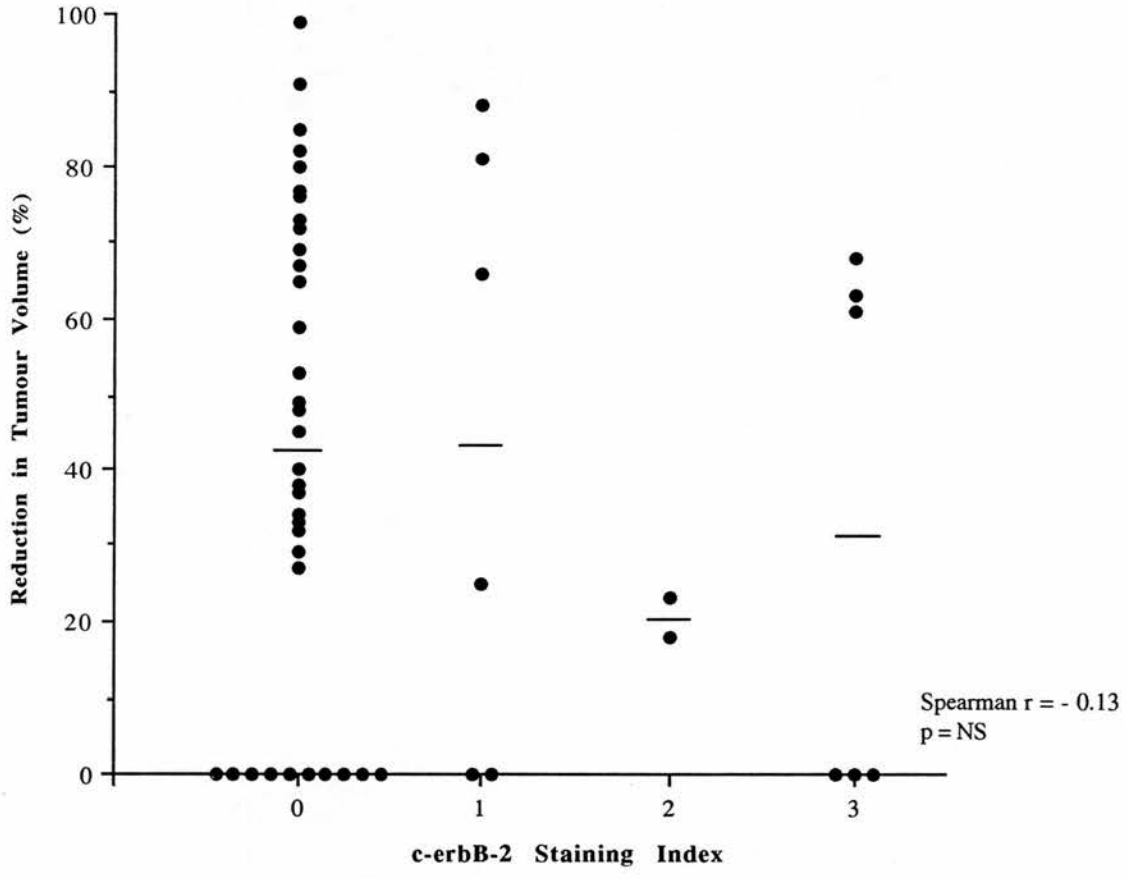


Figure 14.15 shows the relationship between the c-*erb* B-2 staining index, in 51 tumours prior to treatment, and the subsequent response to tamoxifen therapy. The majority of tumours do not express the c-*erb* B-2 protein and in those which do so their is no significant relationship with the response to tamoxifen.

Figure 14.16 The Change in Expression of c-*erb*B-2 and the Response to Tamoxifen

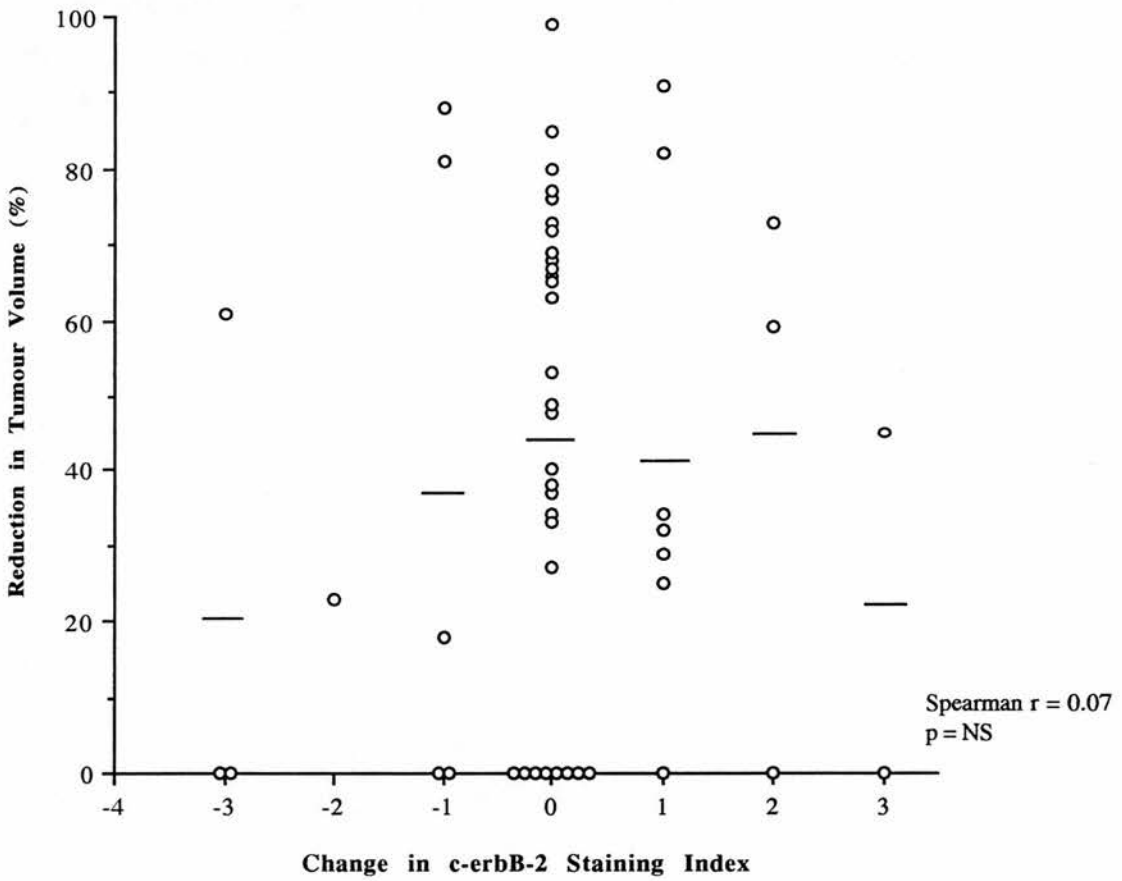


Figure 14.16 shows the relationship between the change in c-*erb* B-2 staining index and the degree of response seen with tamioxifen treatment in 51 patients. There is no significant correlation between these parameters.

14.7 The Expression of *P* -Glycoprotein

The expression of *P* -Glycoprotein has been linked to the MDR phenotype and is one mechanism by which tumour cells may exhibit resistance to several commonly used cytotoxic agents. To examine the possible contribution of this mechanism to tamoxifen resistance, tissue samples of the 52 patients in the study population were stained using immunohistochemical techniques and a polyclonal antibody to *P* -Glycoprotein. A typical positively staining tumour section is shown in Figure 14.17.

Prior to treatment 22 (42%) of the 52 tumours stained positively. There were no significant trends in the degree of staining and subsequent overall response or non-response (Table 14.8a). Interestingly neither of the only two tumours with >75% of cells staining (a staining index of 3) demonstrated a response to tamoxifen. Similarly the degree of staining failed to predict for the degree of response as measured by ultrasound volumes (Figure 14.18). There was, however, a significant trend in the samples taken after treatment in that tumours that had failed to respond displayed higher levels of staining than those which had responded (Table 14.8b). The degree of staining in the post treatment sample was also significantly negatively correlated to the degree of response (Figure 14.19). The increase in staining in non-responding tumours compared to those demonstrating a response was statistically significant both as an overall change and more highly so when the degree of change was calculated (Tables 14.8c and d). There was not, however, a significant correlation between the degree of change in the staining index and the actual measured tumour volume responses (Figure 14.20).

It must be remembered that changes in the staining index can be interpreted in at least two ways. An increase in staining, such as that noted for *P*-Glycoprotein in non-responding patients may be a result of induction of the protein in individual cells or selection of those cells which express the protein and may therefore be resistant to tamoxifen. In either scenario *P*-Glycoprotein appears to be linked to tamoxifen resistance but of course this data cannot prove that it is ultimately responsible.

Figure 14.17 Immunohistochemical Staining for *P*-Glycoprotein

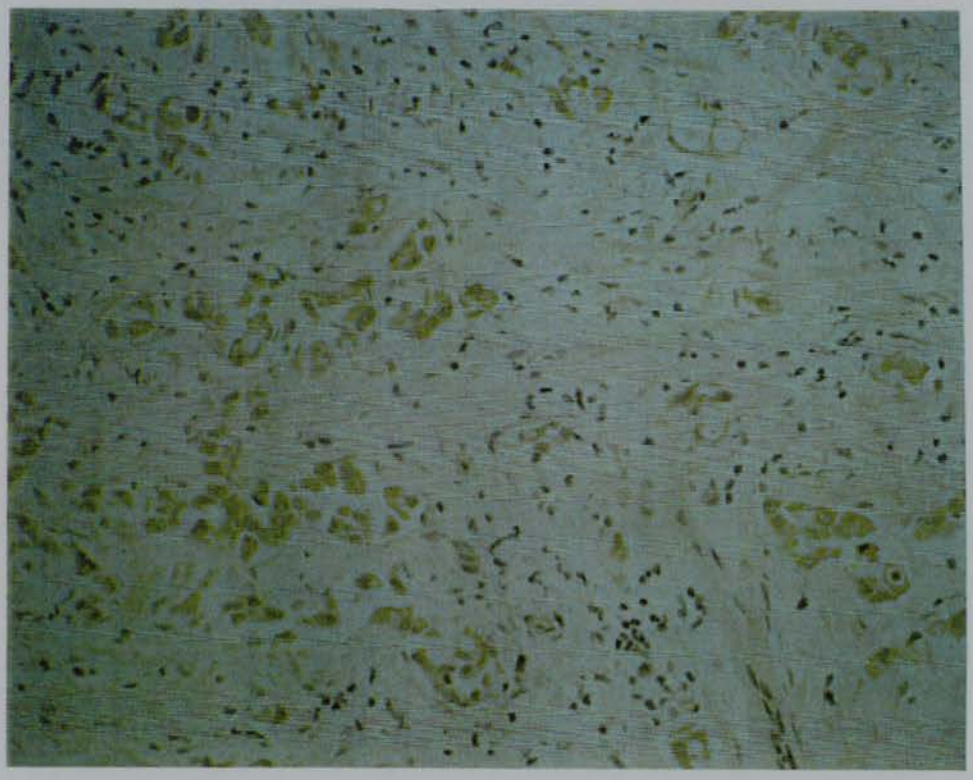


Figure 14.17 shows immunohistochemical staining with polyclonal antibody to *P*-glycoprotein in a section from a primary breast cancer prior to tamoxifen therapy (x40). Note the cytoplasmic staining.

Table 14.8 The Expression of *P*-Glycoprotein and Response to Tamoxifen

a) Before

	Cells Staining			
	<5%	**5-25%**	**25-75%**	**>75%**
Responders (37)	**21**	**9**	**7**	**0**
Non-Responders (15)	**9**	**4**	**0**	**2**

X^2 Trend = 0.03

p = NS

b) After

	Cells Staining			
	<5%	**5-25%**	**25-75%**	**>75%**
Responders (37)	**24**	**8**	**5**	**0**
Non-Responders (15)	**5**	**3**	**4**	**3**

X^2 Trend = 8.61

p = 0.003

c) Change in Expression

	Change in No. of Cells Staining		
	Increase	**No Change**	**Decrease**
Responders(37)	**4**	**26**	**7**
Non-Responders(15)	**8**	**4**	**3**

X^2 Trend = 4.35

p = 0.04

d) Degree of Change in Expression

	Change in Staining Index						
	-3	**-2**	**-1**	**0**	**+1**	**+2**	**+3**
Responders(37)	**0**	**3**	**4**	**25**	**5**	**0**	**0**
Non-Responders(15)	**0**	**0**	**3**	**4**	**4**	**3**	**1**

X^2 Trend = 7.35

p = 0.007

Table 14.8 shows data generated from the immunohistochemical staining, with a polyclonal antibody to *P* -Glycoprotein, of 52 tumours before and after treatment with tamoxifen. Table a) shows no significant trend in the relationship between the degree of staining prior to treatment and the subsequent overall response. The two tumours with the highest degree of staining, however, failed to demonstrate a response. There was a significant trend in the samples taken after treatment (Table b) with higher staining indices in non-responding tumours. The overall and actual changes in staining indices on comparison of the pre- and post-treatment samples were also significantly different in responders and non-responders (Tables c and d). There was a trend towards an increase in staining index in non-responding compared to responding tumours.

Figure 14.18 The Expression of *P*-Glycoprotein Prior to Tamoxifen Therapy and the Subsequent Response

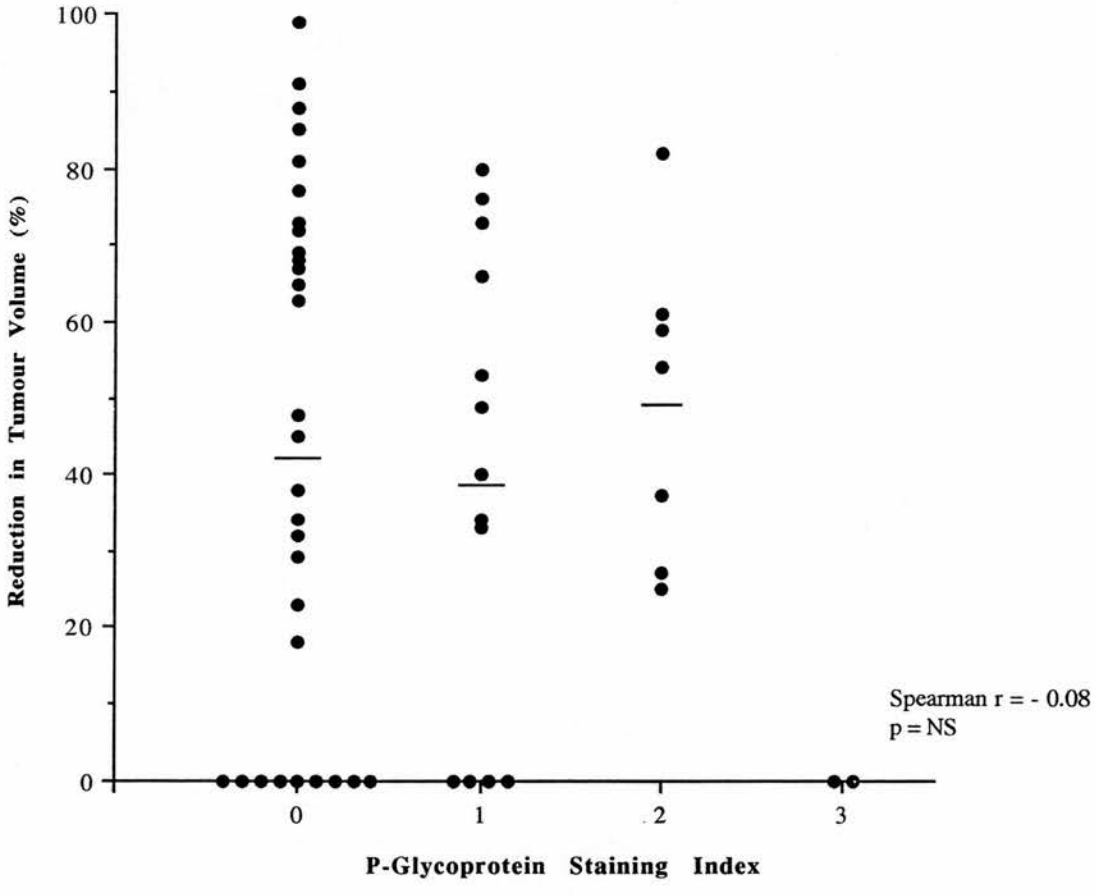


Figure 14.18 shows the relationship, in 52 tumours, between the *P* -Glycoprotein staining index prior to tamoxifen therapy and subsequent response. There is no significant correlation between the two parameters. Note that two tumours had a staining index of 3 and neither responded to tamoxifen.

Figure 14.19 The Expression of *P*-Glycoprotein after Tamoxifen Therapy and the Observed Response

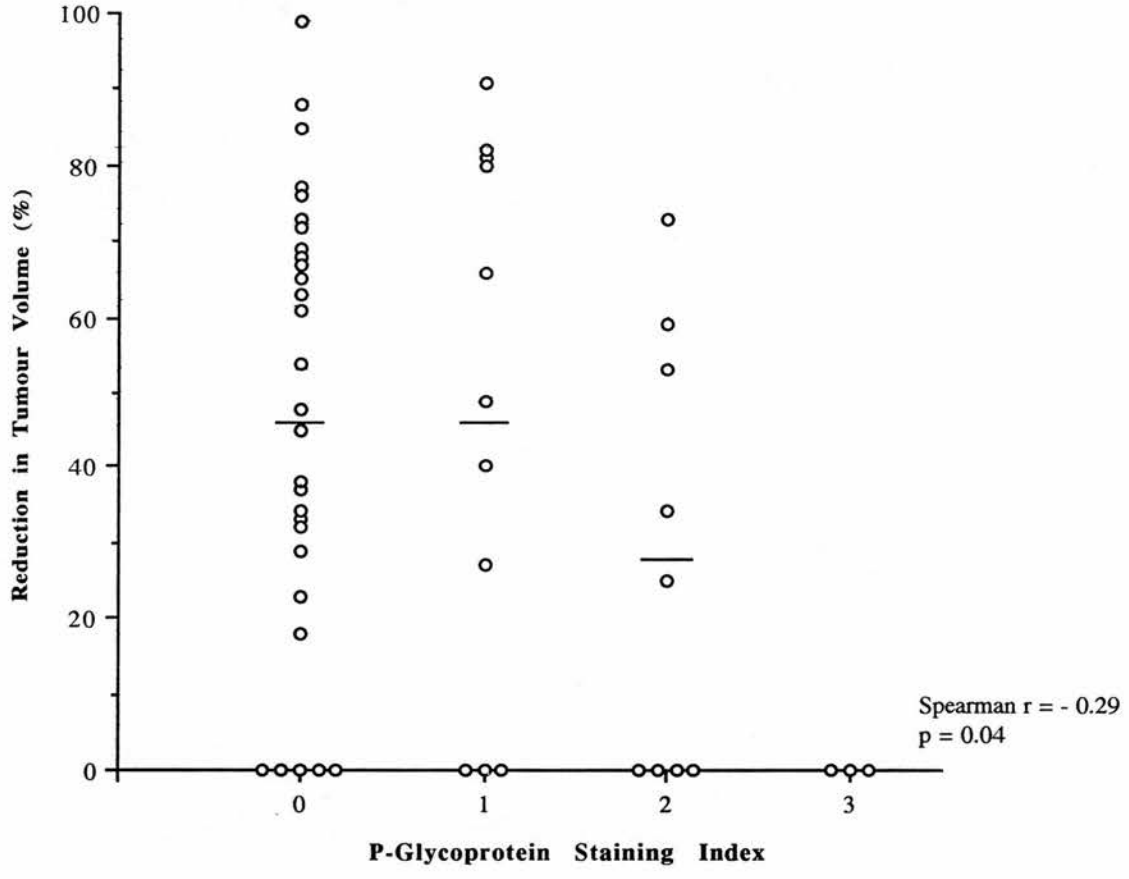


Figure 14.19 shows the relationship between *P* -Glycoprotein staining index of tumour specimens taken <u>after</u> treatment with tamoxifen and the observed response to therapy. There is a significant negative correlation between these parameters with those tumours with higher staining indices displaying smaller clinical responses. Three tumours are represented by the single point with a staining index of 3 and no prior response to tamoxifen.

Figure 14.20 The Change in Expression of *P*-Glycoprotein and the Response to Tamoxifen Therapy

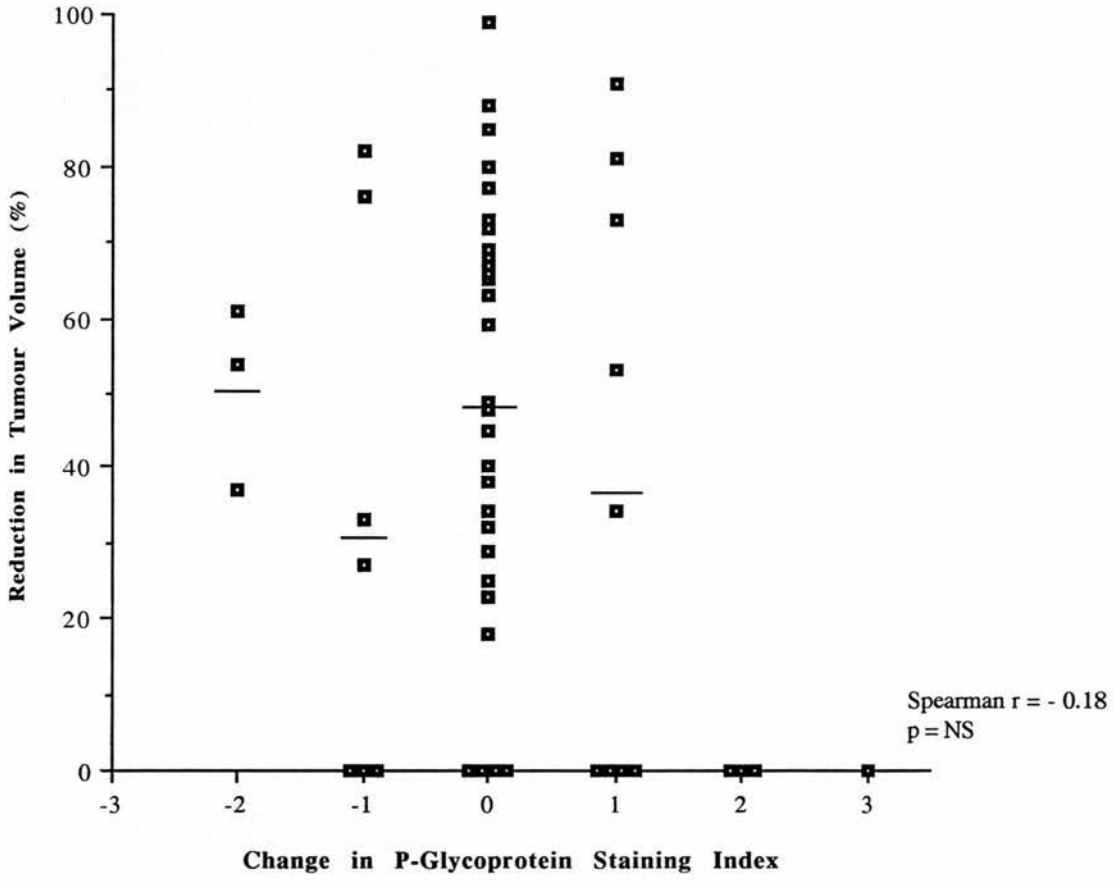


Figure 14.20 shows the relationship between the change in *P* -Glycoprotein staining index with treatment and the clinical response measured. There is no statistically significant correlation between these two parameters in the population studied. None of the four tumours showing a change in staining index of either +2 or +3 demonstrated a response to tamoxifen.

14.8 The Expression of Epidermal Growth Factor Receptor

The tumours in the study population of 52 patients were stained by immunohistochemical techniques to determine the relationship between EGFR expression and the response to tamoxifen. A typical example of tumour tissue staining positively is shown in Figure 14.21. In the tumour samples taken prior to tamoxifen therapy only 12 (23%) stained positive with monoclonal antibody (Table 14.9a). Previously, several authors have reported a negative relationship between the expression of EGFR and the possession of oestrogen recepors (See Capter 5.3). This may explain, therefore, the relatively small number of tumours staining positive in the exclusively ER-positive study group. Indeed, in the pre-treatment samples there was a negative correlation between EGFR staining index and the level of ER expression but this was not statistically significant (Figure 14.22).

EGFR expression has also been suggested as a predictive factor for lack of response to hormone therapy. In this study there appeared to be no association between the degree of staining prior to treament and the overall response to therapy (Table 14.9a). Similarly there was no correlation between staining index and the degree of response as measured by the percentage reduction in tumour volume (Figure 14.23). However, given the limitations of small numbers, there was a highly significant positive correlation between EGFR staining index and the degree of response in those 12 tumours staining positive prior to therapy (Spearman $r = 0.75$, $p= 0.005$).

After treatment with tamoxifen 16 (31%) tumours stained positive for EGFR but with no significant association with the individual responses achieved (Table 14.8b). Similarly there were no significant trends in the change in staining index of tumour samples taken before and after treatment and the observed response (Tables 14.8c and d).

Figure 14.21 Immunohistochemical Staining for EGFR

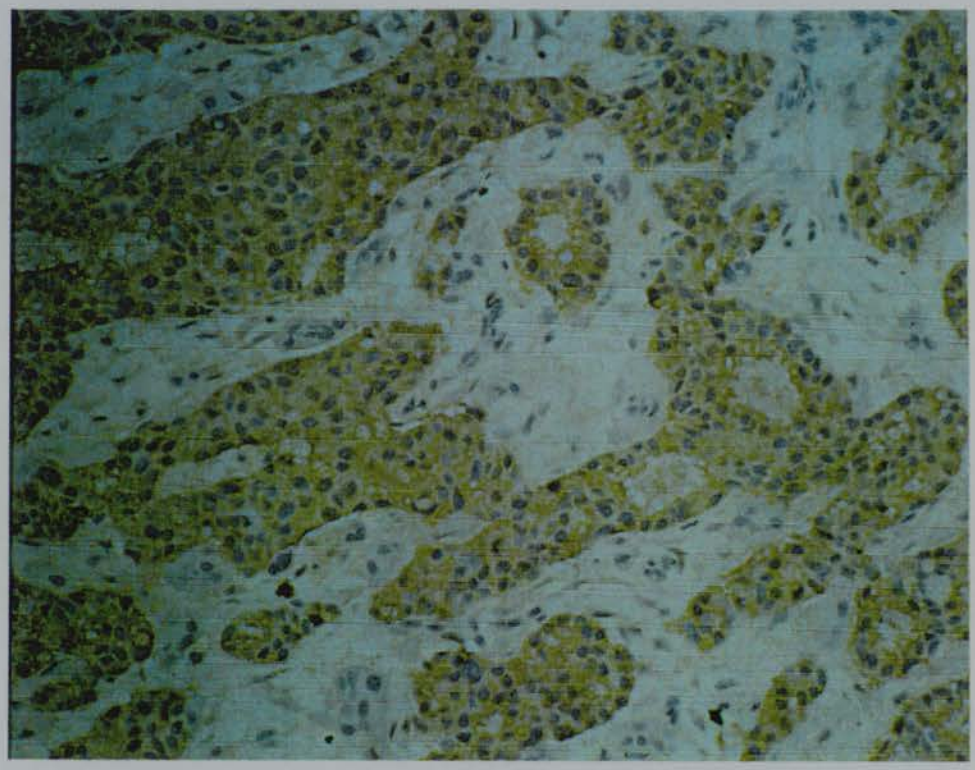


Figure 14.21 shows immunohistochemical staining with monoclonal antibody to EGFR protein in a section from a primary breast cancer prior to tamoxifen therapy (x40). Note the strong membrane staining.

Table 14.9 The Expression of EGFR and Response to Tamoxifen

a) Before

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders (37)	29	2	3	3
Non-Responders (15)	11	4	0	0

X^2 Trend = 0.56

p = NS

b) After

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders (37)	25	5	4	3
Non-Responders (15)	11	1	2	1

X^2 Trend = 0.04

p = NS

c) Overall Change in Expression

	Change in No. of Cells Staining		
	Increase	**No Change**	**Decrease**
Responders(37)	**8**	**23**	**6**
Non-Responders(15)	**4**	**9**	**2**

X^2 Trend = 0.18

p = NS

d) Degree of Change in Expression

	Change in Staining Index						
	-3	**-2**	**-1**	**0**	**+1**	**+2**	**+3**
Responders(37)	**1**	**1**	**4**	**23**	**4**	**2**	**2**
Non-Responders(15)	**0**	**0**	**2**	**9**	**2**	**2**	**0**

X^2 Trend = 0.17

p = NS

Table 14.9 shows data derived from immunohistochemical staining, with EGFR antibody, of tissue sections taken from 52 tumours before and after treatment with tamoxifen. There are no significant trends in sections taken, a) before and b) after treatment on comparison of the degree of staining and observed overall response. There are also no significant trends in either the c) overall or d) the degree of change in staining index with treatment and the observed response.

Figure 14.21 The Expression of EGFR and the ER Content Prior to Tamoxifen Therapy

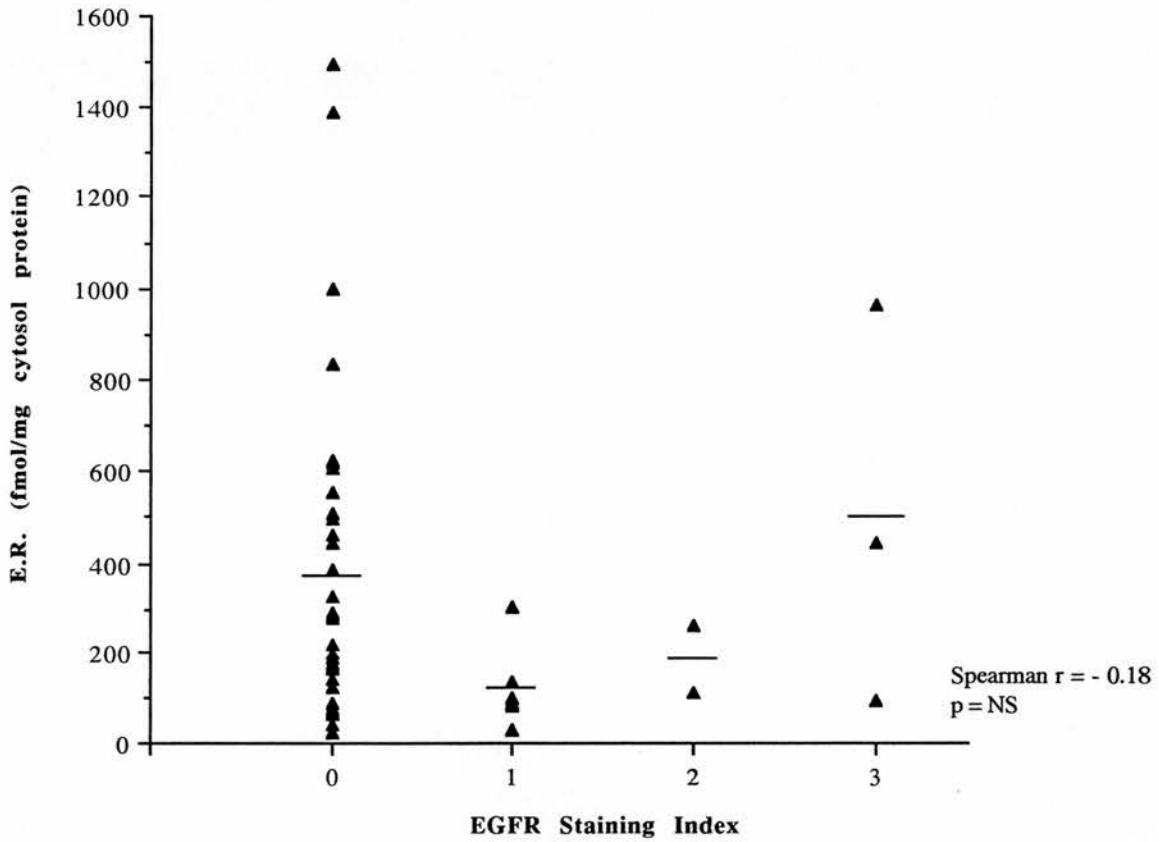


Figure 14.22 shows the relationship between EGFR expression and ER content prior to tamoxifen therapy. The data displayed is for only 51 tumours as ER information was unobtainable for one tumour which in fact expressed EGFR. There is a tendancy to a negative correlation between the two parameters but this fails to reach significance. This is, however, a selected study population of tumours, all of which have an ER > 20 fmol/mg and the vast majority do not express EGFR. There is not a significant correlation between EGFR staining index and ER in those 11 tumours which do stain positive (Spearman r = 0.5377, p = NS) although surprisingly there is a positive correlation coefficient.

Figure 14.23 The Expression of EGFR Prior to Tamoxifen Therapy and the Subsequent Response

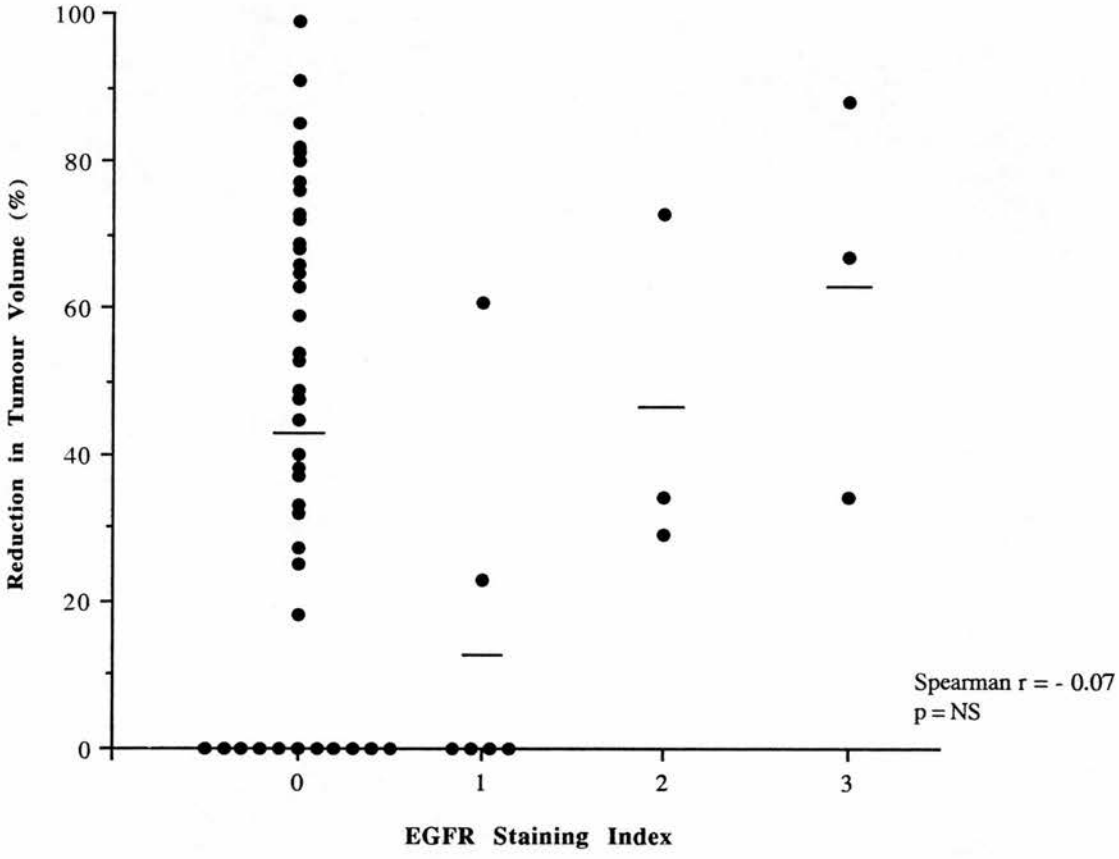


Figure 14.23 shows the relationship between EGFR staining index prior to treatment with tamoxifen and the subsequent observed response. There is no significant correlation between these two parameters in this study population.

14.9 The Expression of p53

In order to study the relationship between the expression of mutant p53 and response to tamoxifen, tumours from the series of 53 patients treated with primary therapy were immunohistochemically assessed. The antibody used in the study detects both mutant and wild type p53 but since the half-life of the wild type antigen is very short it was presumed that the vast majority of the staining observed corresponded to mutant p53. The staining methods involved a microwaving step to aid antigen retrieval and despite repeated attempts interpretable staining (negative or positive) was achieved in samples obtained both before and after treatment in only 44 tumours. An example of positive staining is shown in Figure 14.24. Of the 44 tumours assessed only 5 (11%) showed staining in >5% of tumour cells prior to tamoxifen therapy (Table 14.10a). The same number of tumours were positively staining after treatment although the distribution of the degree of staining was different (Table 14.10b). The small numbers of positively staining tumours makes further assessment of the relationship to response uninformative.

Figure 14.24 Immunohistochemical Staining for p53

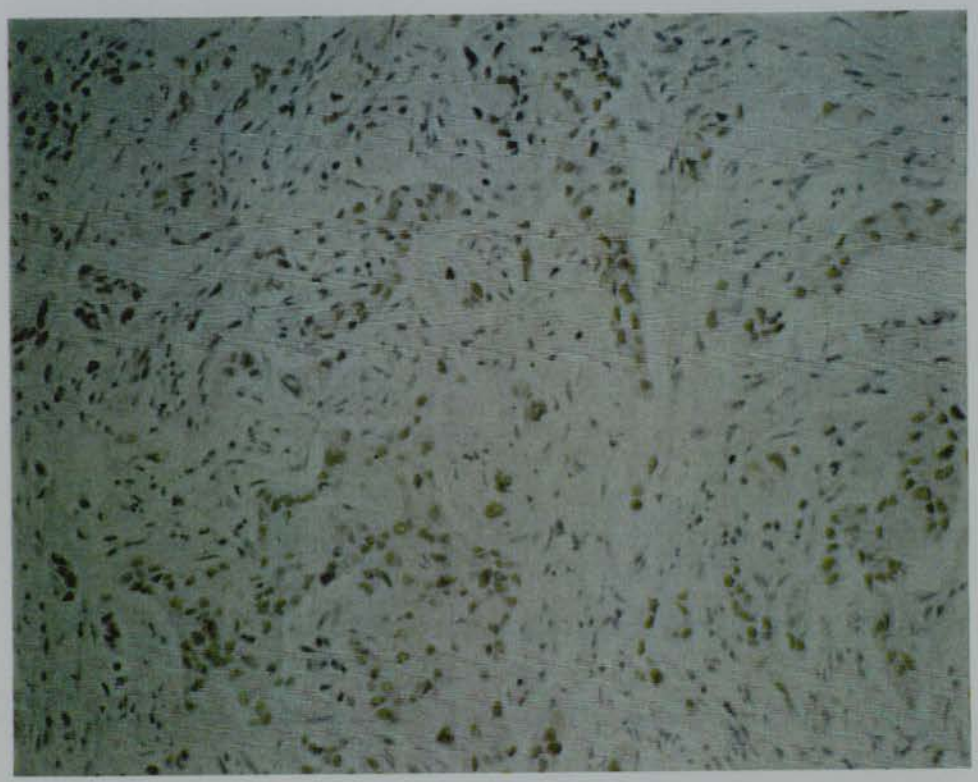


Figure 14.24 shows immunohistochemical staining with monoclonal antibody to p53 protein in a section from a primary breast cancer prior to tamoxifen therapy (x40). Note the strong membrane staining.

Table 14.10 The Expression of p53 and Response to Tamoxifen

a) Before

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders (33)	29	4	0	0
Non-Responders (11)	10	0	0	1

b) After

	Cells Staining			
	<5%	5-25%	25-75%	>75%
Responders (33)	30	1	1	1
Non-Responders (11)	9	0	1	1

X^2 Trend = 1.11

p = NS

Table 14.10 shows the distribution of staining for p53 antigen in responding and non-responding tumours a) prior to and b) after treatment with tamoxifen. There are only very few tumours staining positively for the antigen and there are no significant associations between staining and tumour response.

14.10 Multivariate Analyses of Data Generated by Immunohistochemistry

Multivariate analyses were performed to establish which markers were independently related to the overall and actual degree of response to tamoxifen. The markers included in the analyses were the initial calliper-measured tumour diameter and ultrasound-assessed volume, initial ER content (logarithmically transformed) and the pre- and post- treatment expression of Ki-S1, Bcl-2, pS2, c-*erb*B-2, EGF-R and *P* -Glycoprotein (For working data see Table 14.1). The overall differences in the pre- and post-treatment antigen expression were also modelled (coded as -1 = decrease, 0 = no change, 1 = increase) There were too many missing values to include the p53 and post-treatment ER data. For response multiple logistic regression and for the degree of response multiple normal regression models were fitted. In both cases a stepwise forward variable selection procedure was employed to identify independent predictors of response. Only variables significant at the 5% level were entered and retained in the models. A forward variable selection procedure was performed on patients with all measurements available (48 patients). A final model was then refitted on the maximum possible number of patients.

Two markers were found to be associated with the overall response to tamoxifen, ER measured before treatment and *P* -Glycoprotein expressed after treatment. High values of ER increasing the probability if response and high values of *P* -Glycoprotein having the opposite effect (Table 14.11a).

The degree of response was found to be related to the ER level prior to treatment and the changes in both Ki-S1 and Bcl-2 with tamoxifen therapy. Higher values of ER being associated with greater degrees of response and an increase in either Ki-S1 or Bcl-2 indicating a lower degree of response (Table 14.11b).

Axillary lymph node status, an important prognostic indicator in primary breast cancer, was available for 52 of the patients. In order to examine the relationship between nodal involvement (positive = 1, negative = 0) and the markers listed above along with tumour diameter and volume, a further forward stepwise variable selection procedure was used. None of the markers, their changes on treatment or tumour characteristics were found to be associated with nodal involvement.

Table 14.11 Final Logistic Regression Models for a) Overall and b) Degree of Response and ER, Ki-S1, Bcl-2, pS2, c-*erb*B-2, EGFR and *P* - Glycoprotein.

a)

Variable	Coefficient	O.R.	95% C.I. for O.R.	z	p-value
log ER (pre)	1.44	4.23	1.51 - 11.85	2.74	0.006
P - Gly (post)	-0.91	0.40	0.19 - 0.86	-2.34	0.019
constant	-5.72			-2.21	0.027

b)

Variable	Coefficient	95% C.I. for Coefficient	t	p-value
log ER (pre)	18.19	11.70 - 24.68	5.64	<0.001
Ki-S1 (change)	-12.37	-20.48 - -4.26	-3.07	0.004
Bcl-2 (change)	-14.59	-23.16 - -6.01	-3.42	0.001
constant	-66.63	-101.97 - -31.29	-3.79	<0.001

Table 14.11 shows the results of multivariate analyses to determine those factors which are independently associated with a) overall and b) degree of response. Only those factors which were statistically significant are shown.
O.R. = Odds Ratio, **C.I.** = Confidence Interval.

14.11 Oestrogen Receptor

The relationship of Oestrogen receptor content prior to treatment and the subsequent response to tamoxifen has been demonstrated in Chapter 14 in a group of 95 patients. The strong correlation remains in the selected group of 52 patients described in this chapter and indeed ER has the most significant correlation with the overall and the degree of observed response when subject to multivariate analyses.

ER content post-treatment was only available for 40 tumours and was therefore not included in the analyses. Of these 40 tumours 37 demonstrated decreased levels of ER in the post- compared to the pre-treatment specimens. Univariate analysis by Wilcoxon signed rank sum test found the difference in post- and pre-treatment ER values to be highly significant (z statistic = -5.21, $p < 0.001$).

14.12 Tumours from Patients Treated in the Edinburgh Trial of Primary Systemic Therapy

Material from pre-treatment wedge biopsies and post-treatment definitive loco-regional surgical procedures was available from 25 patients treated according to the protocol of the Edinburgh study. These included 14 patients treated with chemotherapy, six treated with tamoxifen and one treated with both tamoxifen and chemotherapy, all of whom are described above (chapter 13). Four patients have been included, for comparison, from whom archival tumour material was available and who were treated, according to protocol, with the LHRH agonist Goserelin.

It has been previously commented that it is difficult to derive useful information on the biological characteristics of response from the patients included in the study of primary systemic therapy. This is primarily due to the small numbers of patients studied but also due to the very good responses obtained in all but one patient given chemotherapy and two patients given tamoxifen (one of whom did not proceed to definitive surgery). Indeed several patients treated with chemotherapy had insufficient tumour cells in the post-treatment specimens for immunohistochemical investigations. The results obtained are recorded in Table 14.12. With the exception of pS2 and EGFR which should, theoretically, only relate to response to hormonal manipulation, the antibodies used for immunohistochemical analysis of chemotherapy treated tumours were the same as those described above and used in the assessment of tumours treated with tamoxifen.

Only general observations can be made from the data obtained from the fourteen tumours treated with chemotherapy alone:

a) Staining for the proliferation marker Ki-S1 decreased in five out of the nine tumours that responded to treatement from which adequate tumour tissue was available and increased in the one tumour which did not respond.

b) The two patients with strong staining for c-*erb*B-2 have both died during the relatively short period of follow-up

c) Staining for *P*- Glycoprotein was present in only three of thirteen assessable tumours. One of the tumours failed to respond, one tumour, although responding, showed the smallest reduction in tumour volume and the third tumour although responding very well showed a large increase in *P*- Glycoprotein staining and the patient had very early recurrence of metastatic disease.

The six tumours of patients treated with tamoxifen demonstrated staining patterns that appeared to reflect the findings of the large group of tumours reported earlier in the chapter.

The four pre-menopausal patients with ER-positive tumours treated with an LHRH agonist showed interesting staining with pS2 and *P*- Glycoprotein antibodies. All four tumours stained positively for pS2 prior to treatment and negatively after treatement. Two out of the four tumours showed an increase in *P*- Glycoprotein staining with a third tumour showing a high degree of staining before and after treatment.

Table 14.12 Results from Immunohistochemical Analysis of Tumours from Patients Treated in the Edinburgh Trial of Primary Systemic Therapy

Patient No.	Treatment	Sample	Ki-S1	pS2	c-erbB2	pGLY	Bcl-2	EGFR	p53	ER(EIA)	Response
2	C.A.P.	WBx	0		0	0	0		0	10	88%
		Mx	0		0	0	nt		*		
5	C.A.P.	WBx	1		0	0	0		0	0	100%
		WLE	nt		nt	nt	nt		nt		
7	C.A.P.	WBx	0		1	I	3		*	14	58%
		Mx	0		0	0	*		*		
9	C.A.P.	WBx	0		0	1	1		0	19	static
		Mx	2		0	1	0		0		
12	C.A.P.	WBx	0		1	0	0		0	10	96%
		Mx	0		0	0	nt		nt		
15	C.A.P.	WBx	1		0	0	*		0	2	69%
		WLE	0		0	0	*		0		
16	C.A.P.	WBx	2		0	0	3		3	10	96%
		Mx	0		0	1	3		0		
18	C.A.P.	WBx	1		0	0	2		0	12	69%
		Mx	0		0	0	1		0		
20	C.A.P.	WBx	1		0	0	0		3	10	96%
		WLE	0		0	0	1		3		
21	C.A.P.	WBx	0		0	0	*		0	3	90%
		WLE	nt		nt	nt	nt		nt		
b	C.A.P.	WBx	0		3	0	0		0		95%
		Mx	0		0	0	0		0		
c	C.A.P.	WBx	1		3	1	1		0	21	95%
		Mx	0		3	3	0		0		
d	C.A.P.	WBx	1		0	0	1		0	79	73%
		WLE	nt		nt	nt	nt		nt		
e	C.A.P.	WBx	0		0	nt	nt		0		100%
		Mx	nt		nt	nt	nt		nt		
4	TAM+C.A.P.	WBx	0	0	1	1	2		0	108	static
		Mx	0	0	0	0	1		*		
1	TAM	WBx	1	2	0	0	0	0	0	552	87%
		Mx	0	1	0	0	3	0	0		
6	TAM	WBx	0	1	0	1	3	0	0	623	73%
		Mx	0	2	2	2	2	0	0		
13	TAM	WBx	0	0	1	1	1	0	0	45	66%
		Mx	1	0	1	1	1	1	2		
14	TAM	WBx	1	0	0	1	3	0	0	620	40%
		Mx	0	2	0	1	2	0	0		
19	TAM	WBx	1	0	0	0	3	0	0	386	99%
		WLE	0	0	0	0	3	3	0		
a	TAM	WBx	nt	nt	1	2	3	*	*	351	93%
		WLE	0	0	0	0	1	nt	nt		
f	L.H.R.H	WBx	1	2	0	0	0	0	0	394	73%
		Mx	1	0	1	1	1	0	0		
g	L.H.R.H	WBx		3	3	0	3	0	0	42	59%
		Mx	0	0	0	0	1	0	0		
h	L.H.R.H	WBx	1	1	0	1	2	0	*	23	88%
		Mx	0	0	0	3	2	1	0		
i	L.H.R.H	WBx	0	1	2	3	2	0	*	112	**
		Mx	0	0	0	3	1	0	0		

Table 14.12 shows the results obtained from immunohistochemical staining of tumour sections taken before and after primary systemic therapy in a series of 25 patients treated according to the protocol of the Edinburgh Trial.

C.A.P.= Cyclophosphamide, Adriamycin and Prednisolone.

TAM = Tamoxifen.

L.H.R.H. = L.H.R.H. agonist, Goserelin.

WBx = Specimen taken at time of wedge biopsy.

W.L.E. = " " " wide local excision

Mx = " " " mastectomy

Figures in the columns corresponding to each marker visualised represent the staining indeces assigned by the pathologist after examination of tissue sections.

ER(EIA) = Oestrogen receptor content (fmol/mg cytosol protein).

Response = Percentage reduction in tumour volume as assessed by ultrasound.

nt = no tumour cells in tissue section.

* Slides uninterpretable despite repeat staining x2.

** No figures available for the exact response although the tumour had been classified as a "responder" on ultrasound assessment.

Chapter 15: Flow Cytometry and Serial Fine Needle Aspirates

Fine needle aspiration in conjunction with flow cytometric analyses of tumour antigen expression and cell cycle distribution is, potentially, a powerful tool in assessing tumour response to systemic therapy. A subset of patients in both the Elderly Patient's Study and the Trial of Primary Systemic Therapy had serial FNA's taken from their tumours before, after and also, for the majority, during treatment. Patients included were those who had granted permission for additional tumour FNA's to be taken and those for whom it was technically possible for the author to be present to perform the sampling. The results of flow cytometric analyses of antigen expression could then be compared to those obtained from immunohistochemical staining of tissue samples taken before and after treatment from the same tumours (reported in chapter 14). In addition new information from DNA analysis could be considered in relation to the observed tumour responses. The nature of the lymphocyte infiltrate into the tumours was also assessed using antibodies to surface antigens prevalent on Natural Killer (NK) cells (CD56) and B cells (CD19). The presence of both these classes of lymphocyte have been suuggested to relate to therapeutic response. The results from tumours treated with tamoxifen will be considered firstly, followed by those from tumours treated with chemotherapy.

15.1 Tumours Treated with Tamoxifen

The raw data obtained from those tumours treated with tamoxifen is shown in table 15.1. Parameters measured consisted of the lymphocyte markers, expressiuon of c-*erb*B-2, pS2 and Ki-S1, BrdU incorporation and DNA analysis. *P* -Glycoprotein expression was only measured in six non-responding and three responding tumours. The results for antigen expression are listed in two columns headed "%" and "mean". These refer to the two measurements obtainable from analysis of the histograms of fluorescence generated from the flow cytometer. The "%" fluorescence records the percentage of cells staining with a greatrer intensity than background (the control), or loosely, the percentage of cells in the total population expressing the antigen. The "mean" fluorescence records the overall intensity of staining in relation to background, or the amount of antigen expression in a population regardless of the number of cells which express this antigen.

Significant correlations between the parameters measured and the observed response to tamoxifen in samples taken prior to, during and after treatment are shown in Table 15.2 and graphically in Figures 15.1 to 15.9

Table 15.1 Flow Cytometric Assessment of Tumour Samples Obtained by Fine Needle Aspiration Before, During and After Primary Tamoxifen Therapy

Patient No.	Reduction %	Time Point	CD56%	CD19%	P-glyc %	P-glyc Mean	C-erbB2 %	C-erbB2 Mean	pS2 %	pS2 Mean	Ki-S1 %	Ki-S1 Mean	BrdU %	S-Phase %	G0/G1 %	G2+M %	DNA Index	G1 CV
5	static	WBx	6.4	72.6	4.6	0.7	0.9	0.8	1.1	1.0	0.6	1.7	1.1	8.6	91.1	0.3	1.0	2.8
		6 weeks	11.9	50.8	8.2	2.0	0.6	0.7	0.1	0.7	4.0	0.9		16.6	79.5	3.9	1.0	4.1
		Mx	6.7	14.0	0.0	0.5	1.5	1.1	0.6	0.5	0.1	1.2	4.2	6.3	89.0	4.7	1.0	2.7
6	48%	WBx	1.3	14.0			0.0	1.0	0.0	0.8	0.6	0.9	1.3	9.5	68.7	21.8	1.2	5.1
		8 weeks					0.6	1.2	0.4	1.1			1.7	7.3	89.8	2.9	1.0	2.6
		Mx	1.1	2.3			0.4	1.1	1.4	1.2	0.0	0.9	1.5	4.9	86.5	8.6	1.0	3.8
7	88%	WBx	13.3	94.6			28.5	4.6	3.7	1.7	13.9	2.5		4.5	85.9	9.6	1.1	2.8
		8 weeks	1.8	8.7			10.3	3.1	7.9	2.7	7.1	1.3		42.3	54.3	3.4	1.1	3.4
		Mx	0.4	28.7			4.0	2.5	10.6	2.0	4.4	1.4	4.3	5.1	71.3	23.6	1.1	3.4
8	34%	WBx	3.9	9.7			1.1	1.4	0.0	1.2	2.4	0.2	4.4	21.3	77.1	1.6	1.0	3.4
		6 weeks	5.4	16.7			1.0	1.6	1.0	2.5	1.9	0.6		12.0	87.3	0.7	1.0	3.2
		Mx	0.7	1.1			0.0	1.0	0.4	2.2	9.5	1.7	2.8	10.5	76.7	12.8	1.0	4.5
9	static	WBx	5.6	18.1	1.8	1.2	0.6	1.1	1.1	1.0	2.2	1.1	2.2	9.2	84.2	6.6	1.0	2.8
		Mx	1.2	13.3	0.0	0.6	0.0	1.1	1.0	1.5	2.8	1.6	8.4	3.4	82.9	13.7	1.0	4.5
14	33%	WBx	1.9	20.8			0.3	1.1	0.0	0.8	2.3	1.2	3.9	19.0	75.1	5.9	1.7	0.3
		8 weeks	5.9	23.3			1.2	2.0	2.2	1.2			3.4					
		Mx	1.2	8.6			0.1	1.1	3.8	1.4	12.9	0.9	10.8	14.2	81.8	4.0	1.7	1.5
17	63%	WBx	7.8	81.1			16.6	2.4	11.6	0.8	5.1	1.0	1.4	5.5	89.3	5.2	1.6	2.6
		8 weeks	3.5	12.1			10.3	1.3	0.0	1.1				ni			1.5	
		Mx	3.7	5.0			2.0	1.7	0.0	1.6	2.2	1.4	0.4	5.4	84.8	9.8	1.6	2.3
24	29%	WBx	3.9	7.9			9.8	2.0	5.3	0.6	24.0	1.2		1.1	97.1	1.8	1.8	1.9
		8 weeks	0.9	74.8			11.8	1.5	13.1	1.5	8.0	1.8		1.0	92.1	6.9	1.0	3.2
		WLE	5.1	44.6			14.2	1.9	13.5	1.7	1.8	1.8		0.7	94.4	4.9	1.0	4.2

Table 15.1 Continued

Patient No.	Reduction %	Time Point	CD56%	CD19%	P-glyc %	P-glyc Mean	C-erbB2 %	C-erbB2 Mean	pS2 %	pS2 Mean	Ki-S1 %	Ki-S1 Mean	BrdU %	S-Phase %	G0/G1 %	G2+M %	DNA Index	G1 CV
25	49%	WBx	2.8	36.4			22.2	3.6	4.0	0.2	3.9	1.5	1.8	3.8	90.0	6.2	1.0	4.1
		8 weeks	0.1	5.4			9.9	2.1	0.0	1.5			1.0	4.1	84.9	11.0	1.0	3.7
		Mx	3.7	44.2			0.0	1.0	0.0	0.0	0.3	1.1	9.1	4.2	88.3	7.5	1.0	3.9
29	static	WBx	1.4	12.0			1.3	1.5	0.0	1.1	2.4	1.0	ni	1.9	97.9	0.2	1.0	2.1
		6 weeks	1.3	3.5			0.5	1.3	21.4	1.3	7.4	0.9		6.4	89.4	4.2	1.0	2.2
		10 weeks	0.6	4.6			0.4	1.1	1.1	0.9	0.6	1.1		1.5	92.9	5.6	1.0	2.6
		Mx	0.0	8.5			0.0	1.0	0.6	0.6	0.3	1.5	1.8	1.3	95.9	2.8	1.0	3.9
30	72%	WBx	0.7	6.3			16.3	1.5	0.8	0.6	4.5	1.2	0.3	10.2	85.7	4.1	1.8	3.1
		8 weeks	4.7	60.5			3.1	1.4	1.1	0.7	2.3	1.8		29.5	60.5	10.0	1.8	0.6
		Mx	0.7	5.1			2.9	1.8	4.7	1.6	3.1	1.0		7.5	88.6	3.9	1.7	3.8
32	32%	WBx	0.2	0.9	0.3	0.8	2.1	1.3	0.6	4.2	6.5	1.3	2.5	5.8	91.3	2.9	1.0	3.8
		8 weeks	5.9	8.0	2.0	1.3	1.7	1.2	0.9	1.4				12.3	85.1	2.6	1.0	0.4
		Mx	0.2	0.9	1.3	1.0	0.0	0.8	0.0	0.3	3.1	1.2	1.3	11.4	84.6	4.0	2.4	3.7
34	85%	WBx	17.6	93.4	4.3	1.7	3.7	2.6	6.2	1.7	13.7	3.4		5.9	81.6	12.5	1.0	3.7
		Mx	1.9	5.2	5.7	1.4	0.1	0.9	1.3	0.8	1.8	1.2		8.0	84.6	7.4	1.8	2.3
36	static	WBx	5.1	67.0	6.5	1.3	6.2	1.5	0.0	0.3	15.8	2.9		8.0	74.0	18.0	1.6	3.1
		Mx	7.1	45.8	10.7	0.5	2.3	2.3	3.6	1.5	8.9	0.9		ni				
42	76%	WBx	5.3	66.6			17.6	2.9	39.5	3.6	12.6	1.7		2.4	94.4	3.2	2.0	6.1
		4 weeks	0.0	0.0			30.7	2.5	9.8	1.7	0.0	0.9		ni				
		Mx	0.0	22.9			5.1	2.6	18.2	2.3	8.4	2.2		11.6	84.1	4.3	2.0	1.6
44	prog	WBx	2.5	16.2			0.2	1.0	0.0	1.3	0.0	1.0	1.5	12.4	85.0	2.6	1.0	3.1
		8 weeks	1.2	3.8			1.6	1.3	2.8	1.2			2.4	8.7	88.6	2.7	1.0	3.8

Table 15.1 Continued

Patient No.	Reduction %	Time Point	CD56%	CD19%	P-glyc %	P-glyc Mean	C-erbB2 %	C-erbB2 Mean	pS2 %	pS2 Mean	Ki-S1 %	Ki-S1 Mean	BrdU %	S-Phase %	G0/G1 %	G2+M %	DNA Index	G1 CV
44		Mx	3.2	20.1			0.0	1.0	0.0	0.7	0.0	0.7	0.6	ni			1.3	
45	static	WBx	0.4	60.9	5.1	0.9	0.4	1.3	5.2	1.3	44.2	0.6		6.1	85.8	8.1	2.0	2.0
		8 weeks	4.7	32.8	6.1	0.8	1.1	0.4	0.4	0.4	0.0	1.5		29.9	65.6	4.5	2.1	2.8
		Mx	11.9	57.8	2.4	1.2	2.7	1.4	1.0	1.0	0.9	0.8		32.2	65.9	1.9	2.0	1.9
51	77%	WBX	11.6	87.5			18.3	2.2	11.6	0.7	3.0	1.5	7.4	9.4	85.4	5.2	1.6	2.7
		4 weeks	7.0	68.3			10.5	1.5	23.2	0.6	0.5	1.2	1.3	4.1	90.4	5.5	1.6	2.2
		10 weeks	6.9	69.4			17.8	2.5	10.2	1.4	0.1	1.4	2.2	1.1	90.9	8.0	1.6	1.1
		Mx	1.4	22.6			2.1	1.6	1.7	1.3	0.7	1.7	1.0	3.4	86.4	10.2	1.8	2.2
52	66%	WBx	5.2	7.9	0.9	1.2	3.6	2.0	0.0	2.3	0.5	0.6	2.2	7.6	83.9	8.5	1.6	2.0
		6 weeks	3.8	8.1	3.2	4.8	1.3	1.9	0.6	11.9				3.1	87.5	9.4	*	
		Mx	2.4	22.2	0.7	1.0	20.3	2.4	0.8	1.6	5.1	3.0	1.6	10.7	83.1	6.2	1.6	1.6
53	73%	WBx	2.4	46.1			9.5	1.9	29.6	4.0	4.8	1.4	1.1	4.9	87.6	7.5	1.0	3.8
		6 weeks	6.0	35.0			4.7	1.5	21.3	3.5	0.0	2.3		3.3	91.1	5.6	1.0	2.8
		Mx	1.8	76.0			5.1	1.4	18.4	1.9	14.4	2.8		2.0	90.8	7.2	1.0	2.3
i	static	WBx	5.5	31.3	15.0	2.1	3.6	2.2	0.0	1.3	2.9	0.8	2.8	15.1	64.1	20.8	1.0	0.3
		4 weeks	1.3	7.4	6.3	1.4	0.7	1.2	0.0	0.8	0.0	1.1		4.5	93.7	1.8	1.0	2.9
		8 weeks	4.3	27.2	0.7	1.1	11.6	2.3	2.0	2.6	0.0	0.5	1.5	6.4	88.3	5.3	1.0	3.1
		12 weeks	12.6	18.3	24.0	1.4	6.0	1.7	0.0	1.1	0.8	1.2		10.2	80.5	9.3	1.0	2.9
		Chemo 1	3.4	41.8	25.3	1.6	8.0	1.5	0.0	1.3	0.0	1.1	2.8	22.2	77.5	0.3	1.0	2.7
ii	static	WBx	2.6	31.7	2.6	1.1	0.4	0.5	0.0	0.3	0.0	1.0	9.5	18.4	77.4	4.2	2.0	1.2
		6 weeks	1.4	15.7	11.1	0.7	14.2	1.6	0.8	1.0	5.5	1.6						
		10 weeks	3.5	25.3	2.5	3.5	8.8	2.7	1.8	1.5	3.8	1.6		29.3	68.6	2.1	2.0	2.0

Table 15.1 shows results obtained from flow cytometric analysis of tumour samples obtained by fine needle aspiration before, after and, in some cases, during tamoxifen treatment.

Reduction = Reduction in tumour volume as assessed by ultrasound.

WBx = FNA taken at time of pre-treatment tumour wedge biopsy.

Mx/WLE = FNA taken at time of post-treatment mastectomy/wide local excision.

Figures for each marker studied are expressed as the percentage of events (cells) staining with fluorescence greater than background or the ratio of the means of the test sample over the background.

Figures for cell cycle analyses were calculated by the computer software as previously described.

ni = DNA histogram **n**ot **i**nterpretable.

Table 15.2 Significant Correlations Between Marker Expression i) Prior to ii) During and iii) After Treatment and Final % Tumour Volume Reduction

i) Prior to Tamoxifen

Marker	Spearman r value	p value
In all tumours		
c-*erb*B-2 (%)	0.654	0.001
c-*erb*B-2 (mean)	0.674	0.0006
pS2 (%)	0.554	0.008
Responding tumours only		
Ki-S1 (mean)	0.639	0.014
CD 56 (%)	0.660	0.01
CD 19 (%)	0.744	0.002

ii) During Treatment

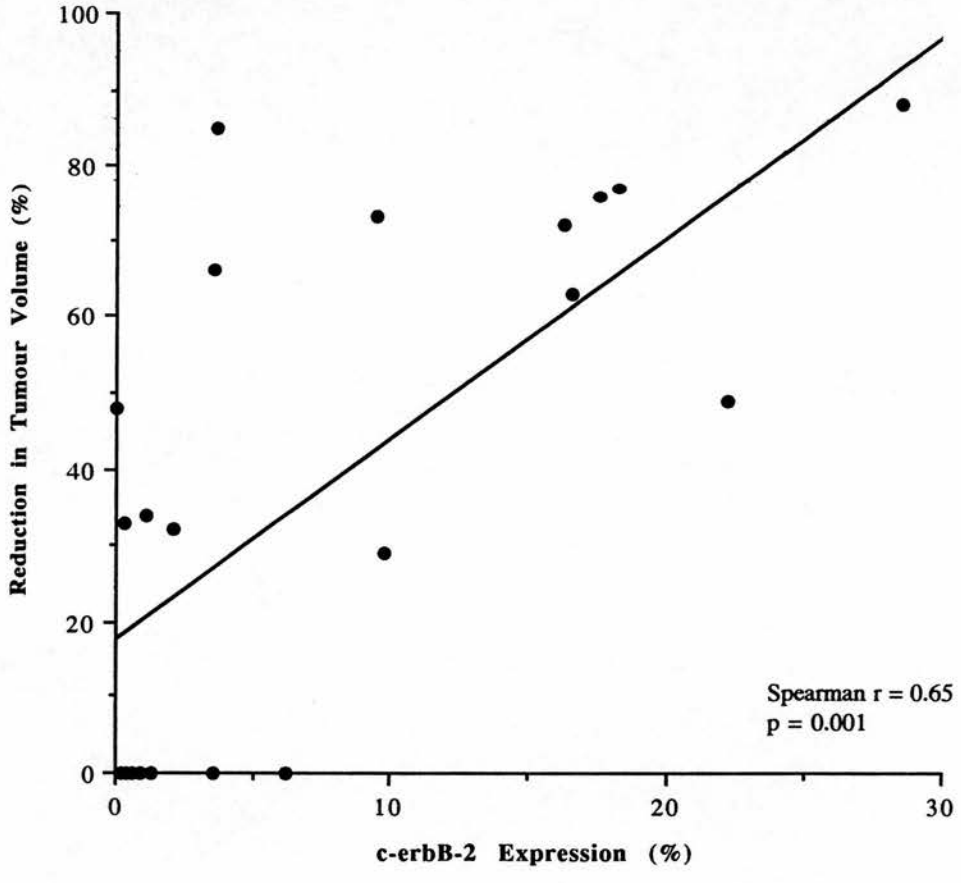
c-*erb*B-2 (mean)	0.468	0.043

iii) After Tamoxifen

All tumours		
pS2 (mean)	0.480	0.028
CD56 (%)	- 0.504	0.017

Figure 15.1 The Relationship Between i) Percentage and ii) Mean Cellular C-*erb*B-2 Expression and Reduction in Tumour Volume

i)



ii)

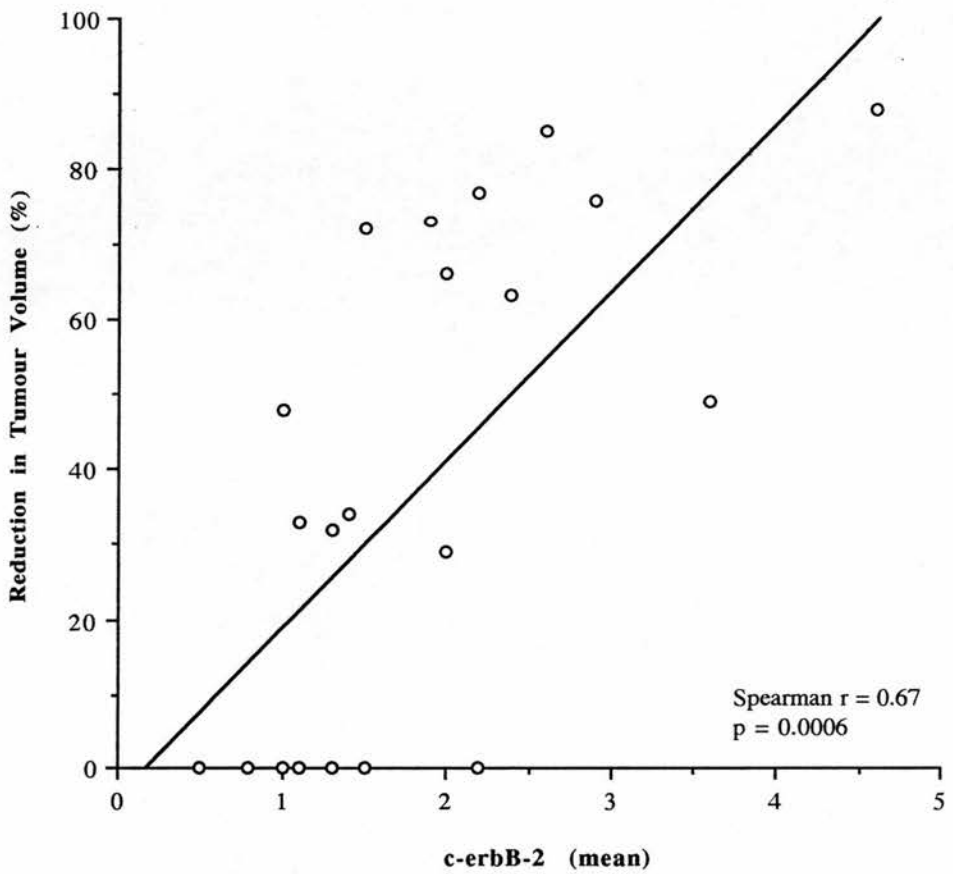


Figure 15.1 shows the relationship between the observed reduction in tumour volume and i) the percentage of tumour cells expressing c-*erb* B-2 and ii) the mean fluorescence (as compared to background and expressed as a ratio), in FNA's of 22 tumours prior to treatment. Both correlations are statistically significant although that between volume reduction and mean level of c-*erb*B-2 expression is better.

Figure 15.2 The Relationship Between Percentage Cellular pS2 Expression Pre-Treatment and Reduction in Tumour Volume

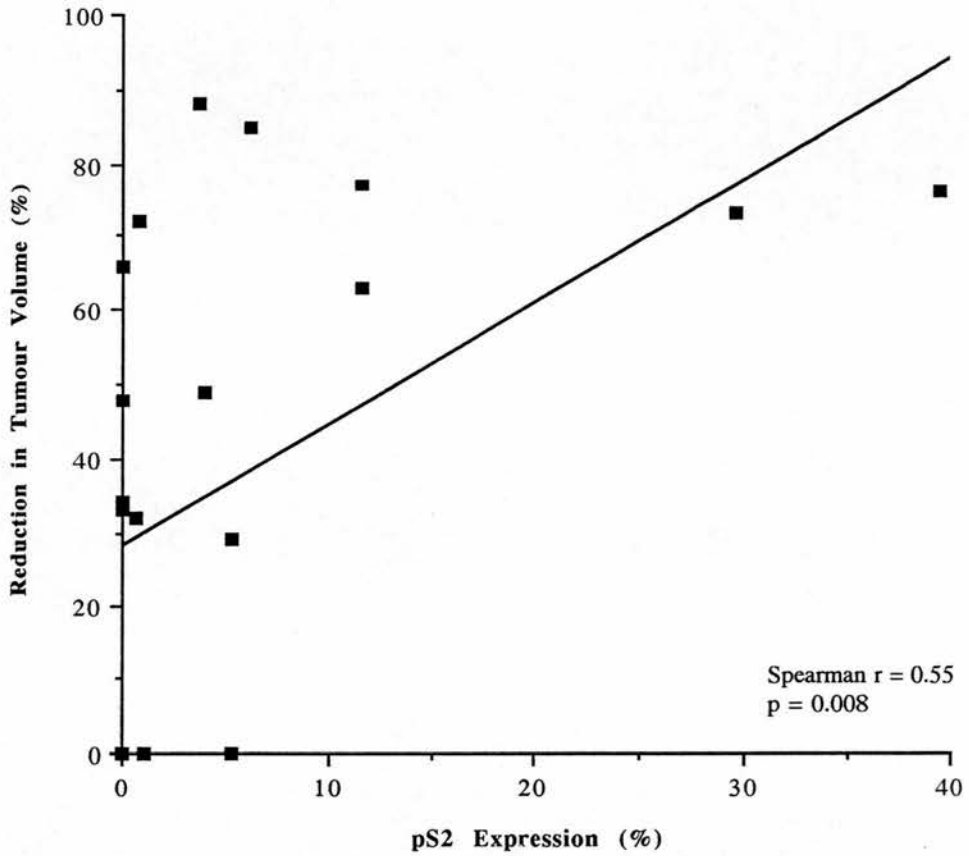


Figure 15.2 shows the statistically significant correlation between pS2 expression prior to treatment and the subsequent ultrasound-assessed reduction in tumour volume after three months tamoxifen therapy. Prior to treatment only the percentage of cells showing fluorescence greater than background showed a significant correlation not the mean level of flourescence (= mean cellular expression)

Figure 15.3 The Relationship Between Mean Cellular Ki-S1 Expression Pre-Treatment and Reduction in Tumour Volume in Responding Tumours Only

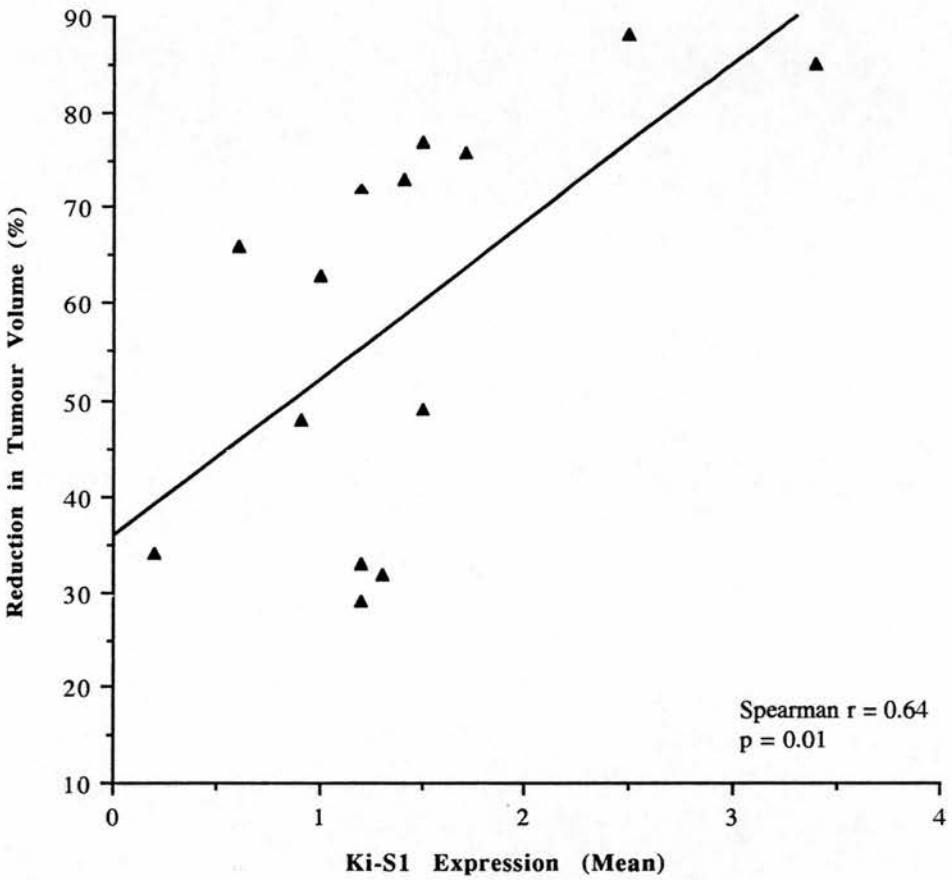


Figure 15.3 shows the relationship between tumour response and mean fluorescence levels (as compared to background and expressed as a ratio) in cells stained with Ki-S1 antibody taken by FNA prior to treatment. There was not a significant relationship when the whole population was included and the above data represents responding patients only.

i) Samples Taken Prior to Treatment

The above table and figures illustrate only the significant correlations obtained when all the parameters measured by flow cytometry were tested as predictive factors for tumour response. Interestingly c-*erb*B-2 expression as determined both by the percentage and mean cellular expression was a strong predictive factor for the degree of response to tamoxifen (Figure 15.1). Both parameters of the measurement of c-*erb*B-2 expression were also significantly related to overall response with responders having higher levels than non-responders (percentage cellular expression: MWU statistic = 22.5, p = 0.024 and mean cellular expression: MWU statistic = 22.0, p = 0.022). This had not been demonstrated in the immunohistochemical studies reported in Chapter 14. Indeed when the relationship between immunohistochemical and flow cytometric staining for c-*erb*B-2 in the same tumours was tested statistically it was not significant. The percentage of cells staining with pS2 antibody but not the mean levels of staining was predictive of the degree of response (Figure 15.2). The relationship with overall response just failed to reach statistical significance (MWU statistic = 30, p = 0.08). A relationship with response had not been demonstrated in the immunohistochemical studies and, in a similar fashion to c-*erb*B-2, there was not a statistically significant correlation between the two methods of pS2 analyses when these were formally tested.

Cellular proliferation prior to treament as determined by S-phase fraction, BrdU uptake and percentage cellular expression of Ki-S1 was not significantly related to the reduction in tumour volume. However, the mean cellular expression of Ki-S1 did correlate with the degree of response but only in responding tumours (Figure 15.3)

Other information obtained from DNA analysis also failed to reveal any significant associations with overall or actual degree of tumour response. In terms of ploidy only one of nine aneuploid tumours did not respond wheras five of ten diploid tumours failed to show a response. Three tumours could be classified as tetrapoid prior to treatment, one of which had a subsequent response.

The percentage of cells, in pre-treatment FNA samples, expressing the leucocyte antigens CD19 (B lymphocytes) and CD56 (Natural Killer cells) both correlated positively with the degree of response when assessed in the sub-population of responding tumours only (Figure 15.4).

ii) Samples Taken During Treatment

Midpoint samples (six to eight weeks into treatment) were available from 19 of the 22

patients studied. When these samples were analysed in terms of correlation with final degree of response only the mean cellular fluorescence of c-*erb*B-2 remained significant. All other parameters had lost statistical significance.

iii) Samples Taken After Treatment

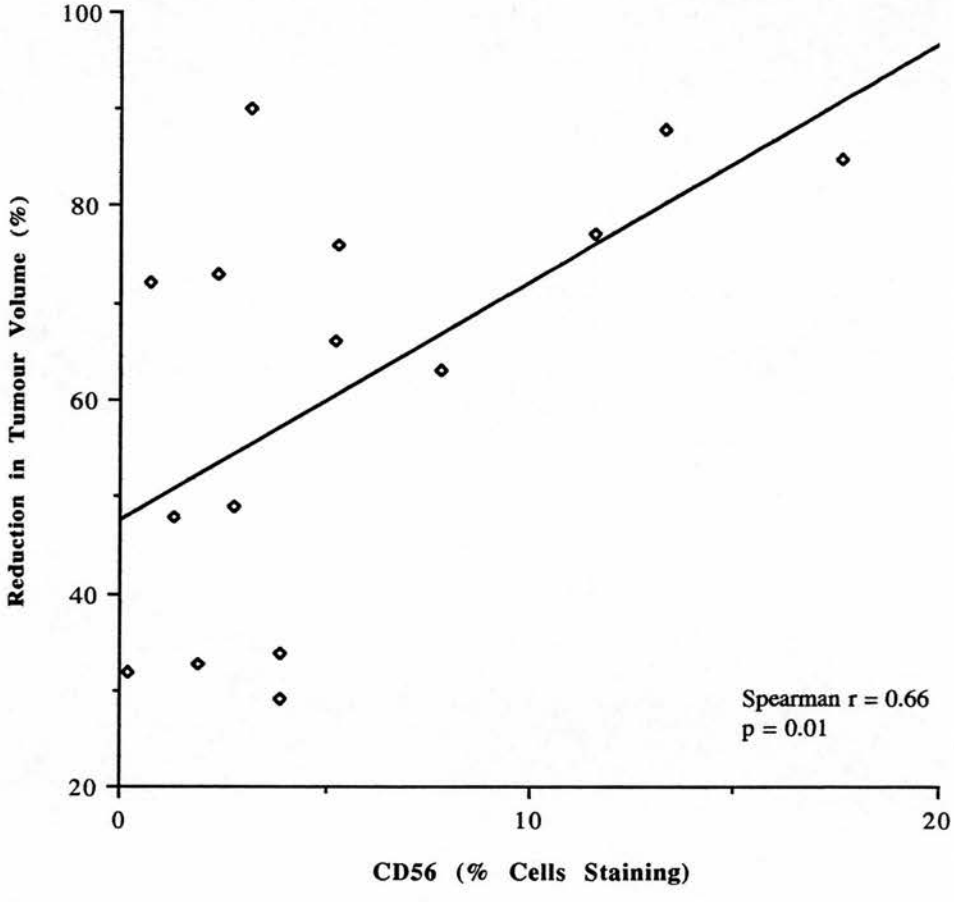
In samples taken after treatment there were significant correlations between the degree of the observed prior response and mean pS2 expression (and not percentage as seen in the pre-treatment samples) and percentage of cells expressing CD56 (negative Spearman r value). These results imply greater levels of pS2, although not larger numbers of cells actually expressing pS2, in tumours that have responded best to treatment and also a greater reduction in the number of NK cells in these sensitive tumours after tamoxifen therapy. The levels of NK cells were positively correlated with the degree of response in pre-treatment samples.

Changes in Marker Expression with Treatment

When the levels of expression, of the various markers examined, were compared before, during and after treatment for individual tumours few significant trends were found (Table 15.2). There was a significant decrease in cellular expression of c-*erb*B-2 (both percentage and mean fluorescence) on comparison of the before and after samples taken from responding tumours (but not non-responders). Likewise there were significant decreases in CD56 expressing cells in responding as opposed to non-responding tumours. However there was a significant decrease in CD19 cells in only non-responding tumours. There was no significant change in these three markers in the midpoint samples and such changes, therefore, could not have been picked up earlier in treatment. There was, however, a significant decrease in mean Ki-S1 expression between the pre-treatment and the midpoint sample in responding tumours. DNA analysis of serial samples from individual tumours failed to show any significant trends, in particular no changes in S-phase or G_0/G_1 phase. Three tumours showed the development of aneuploid peaks during treatment and one aneuploid tumour became diploid.

Figure 15.4 The Relationship Between Response and Lymphocyte Markers i) CD56 and ii) CD19 in Responding Tumours Only

i)



ii)

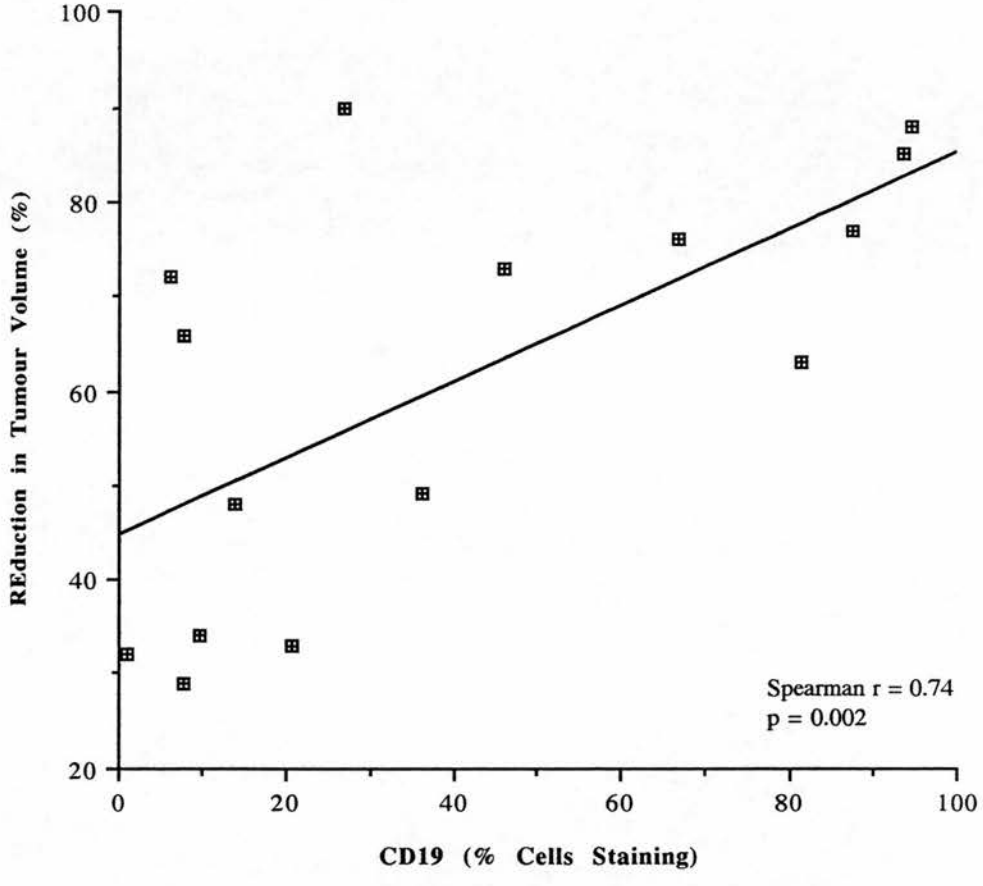


Figure 15.4 shows the correlations in pre-treatment FNA's between subsequent tumour response and levels of Natural Killer cell and B lymphocyte infiltrate as estimated by the percentage of cells expressing i) CD56 and ii) CD19 surface markers. There are statistically significant correlations for both markers.

Table 15.2 Significant Changes in Marker Expression With Treatment

Marker	Samples Compared	WSR Test "p value"
i) Responding Tumours		
c-*erb*B-2 (%)	WBx and Mx/WLE	0.045
c-*erb*B-2 (Mean)	WBx and Mx/WLE	0.016
Ki-S1 (Mean)	WBx and Midpoint	0.016
CD56 (%)	WBx and Mx/WLE	0.024
ii) Non-Responding Tumours		
CD19 (%)	WBx and Mx/WLE	0.042

Table 15.2 shows only the statistically significant changes in expression of the markers tested when samples taken before, during and after treatment of individual tumours were compared. All parameters displayed above decreased with treatment.

WSR = Wilcoxon Signed Rank Test
WBx = FNA taken at time of Wedge Biopsy
Mx/WLE = FNA taken at time of Mastectomy /Wide Local Excision

Relationships Between Different Markers

There were several significant correlations between the different markers studied on samples obtained from individual tumours prior to treatment. These are listed below;

a) c-*erb*B-2 (%) and Ki-S1 (mean), Spearman r = 0.540, p = 0.01

b) c-*erb*B-2 (%) and CD56 (%), Spearman r = 0.458, p = 0.032

c) c-*erb*B-2 (%) and CD19 (%), Spearman r = 0.374, P = 0.002

d) CD56 (%) and CD 19 (%), Spearman r = 0.619, p < 0.0001

e) pS2 (%) and CD19 (%), Spearman r = 0.520, p = 0.013

f) Ki-S1 (mean) and CD19 (%), Spearman r = 0.557, p = 0.007

Of interest, given the results of the work reported on the relationships between proliferative markers in preceeding chapters, the correlation between BrdU incorporation and S-phase just failed to reach statistical significance (Spearman r = 0.494, p = 0.061 in FNA's taken prior to treatment). There were no significant relationships between Ki-S1, BrdU incorporation and S-phase.

15.2 Tumours Treated with Chemotherapy

Data obtained from flow cytometric assessment of FNA samples taken from tumours treated as per the protocol of the Edinburgh Trial of Systemic Therapy is shown in Table 15.3. The data is limited by the very few numbers of tumours from which samples were available and by the narrow range of responses measured by ultrasound. Only one tumour failed to respond to treatment and the vast majority of the responders displayed a reduction in tumour volume of greater than 90%. Four tumours could be sampled four times; at wedge biopsy, at the first and third administrations of chemotherapy and at the time of definitive surgery. The other tumours listed were sampled at the time of the first treatment with chemotherapy but with rapid responses became impalpable or yielded unanalysable material on FNA. It was possible, however, to sample some of these tumours after treatment under direct vision at the time of definitive surgery.

There were no significant correlations between the degree of response and any of the parameters measured at any time. Neither did there appear to be any distinguishing features of the sample obtained prior to treatment of the one tumour that failed to respond to treatment.

Adriamycin uptake was measured in FNA samples taken one hour after the completion of intra-venous infusion of the drug. The appearance of one such sample, viewed under a fluorescence microscope, is shown in Figure 15.5. The intrinsic fluorescence of adriamycin could also be measured by the flow cytometer and expressed as the ratio of the mean fluorescence of the post infusion sample and the pre-infusion sample as shown in Figure 15.6. There was no correlation between adriamycin uptake and the degree of response or any other parameter, including *P*-glycoprotein expression, although numbers were limited.

Of the four tumours that were sampled at four time points, two produced interesting series of DNA histograms (Figures 15.7 and 15.8). The tumour from patient 20 was diploid and the changes in the cell cycle distribution with treatment are entirely in keeping with previous work suggesting that adriamycin induces a G_2 block. The tumour from patient 18 was initially diploid, prior to treatment, but an aneuploid subpopulation is seen to develop during treatment. This tumour was shown to express *P* -glycoprotein but on dual staining it was the diploid and not this new aneuploid population that was predominantly responsible for the staining.

There were no significant differences between parameters measured prior to, and after treatment, although only seven tumours had analysable data from these two time points.

Table 15.3 Flow Cytometric Assessment of Tumour Samples Obtained by Fine Needle Aspiration Before, During and After Primary Chemotherapy

Patient No.	Reduction %	Time Point	CD56%	CD19%	P-glyc%	P-glyc Mean	Ki-S1 %	Ki-S1 Mean	BrdU %	G0/G1 %	S-Phase %	G2+M %	DNA Index	G1 CV	Adr Mean
2	88%	Wbx	1.5	57.7	4.4	1.7	11.6	2.8	8.3	79.6	18.5	1.9	1.9	3.0	
		Pre Chemo 1	3.1	23.3	7.3	2.1	17.7	1.6	5.7	77.5	13.4	9.1	2.0	0.3	
		Post chemo 1							10.7	79.7	8.3	12.0	2.0	4.0	1.0
7	58%	Pre Chemo 1	0.7	4.9	0.0		0.0	0.9	2.3	81.8	17.0	1.2	1.0	1.9	
		Post Chemo 1							1.8						
9	Static	Pre Chemo 1	5.0	29.3	5.4	1.2	1.3	1.3	1.6	90.7	8.4	0.9	1.0	2.9	1.1
		Mx	0.7	28.2	7.7	0.5	10.6	1.6	3.8	73.4	17.9	8.7	1.0	2.0	
10	95%	Pre Chemo 1	0.1	25.4	3.6	1.5	0.0	0.9	*	*	*	*	1.4	*	0.7
		Mx	0.5	14.1	3.2	1.8	1.0	1.3	0.7	92.9	6.7	0.4	1.0	3.3	
12	96%	WBx	1.2	52.5	0.0	0.8	0.9	1.0		79.1	11.8	9.1	1.7	2.2	
		Pre Chemo 1	0.1	10.9	0.0	1.0	1.9	1.1		70.7	20.0	9.3	1.6	2.9	0.6
		Pre Chemo 3	2.2	9.4	0.0	1.1	0.0	0.7		*	*	*	*	*	1.1
		Mx	1.4	35.2	0.0	1.4	1.1	1.1		53.5	45.8	0.7	1.6	3.6	
15	69%	WBx	1.5	47.7	1.4	1.6	12.0	1.3	3.8	69.1	22.5	8.4	1.6	3.8	
		Pre Chemo 1	2.7	38.1	0.0	1.2	2.1	0.8	22.3	64.1	25.8	10.1	1.7	3.9	4.2
		Pre Chemo 3	2.9	15.1	1.2	2.7	0.0	1.7	2.2	*	*	*	1.6	*	0.6
		WLE	6.8	28.1	5.0	2.4	8.9	1.2	5.6	76.4	16.7	6.9	1.7	3.8	
16	96%	WBx	3.2	59.0	4.2	1.2	0.0	1.1		69.1	23.6	7.3	1.5	5.2	
		Mx	0.9	31.7	7.1	2.9	20.8	1.3		70.1	22.7	7.2	1.7	3.2	

Table 15.3 Continued

Patient No.	Reduction %	Time Point	CD56%	CD19%	P-glyc%	P-glyc Mean	Ki-S1 %	Ki-S1 Mean	BrdU %	G0/G1 %	S-Phase %	G2+M %	DNA Index	G1 CV	Adr Mean
18	69%	WBx	0.2	23.5	3.3	1.6	3.6	1.4	0.6	75.6	5.7	18.7	1.0	3.1	
		Pre Chemo 1	0.9	31.0	4.7	6.1	1.2	1.4	1.6	72.3	5.7	22.0	1.0	3.6	5.4
		Pre Chemo 3	1.8	22.3	0.0	0.9	0.6	1.1	0.9	*	*	*	*	*	0.9
		Mx	3.0	39.6	1.1	1.2	0.2	1.1	0.7	70.2	13.4	16.4	1.74**	3.3	
20	96%	WBx	3.5	3.0	3.7	2.1	1.7	1.5	*	86.9	6.6	6.5	1.0	3.9	
		Pre Chemo 1	0.0	5.5	0.8	1.0	0.7	1.1	2.1	76.1	14.3	9.6	1.0	4.2	0.8
		Pre Chemo 3	3.0	17.3	7.6	4.8	0.0	0.6	0.7	77.6	2.7	19.7	1.0	4.6	0.3
		WLE	3.7	19.3	5.8	2.6	1.7	1.4	0.4	67.8	4.4	27.8	1.0	4.0	
21	90%	WBx	0.0	72.9	6.4	1.1	4.3	1.3	1.1	81.2	12.3	6.5	1.0	2.8	
b	95%	Pre Chemo 1	1.3	33.5	0.0	0.8	23.6	2.3	2.1	54.2	33.4	12.4	1.4	2.5	
		Post Chemo 1							53	61.4	21.2	17.4	1.4	3.2	1.5
c	95%	Pre Chemo 1	0.2	4.8	2.5	1.2	0.5	0.6	*	96.6	1.2	2.2	1.0	2.2	1.3
d	100%	Pre Chemo 1	0.0	0.0	11.7	1.2	6.2	0.8	3.4	81.4	11.8	6.8	1.0	1.6	1.4
		Pre Chemo 3	0.0	0.0	30.2	0.8	0.0	0.8	*	84.3	13.1	2.6	1.0	2.3	2.2
e	99%	Wbx	0.0	35.7	1.0	1.2	0.0	0.7	6.5	71.2	18.9	9.9	1.6	3.5	
		Pre Chemo 1	0.4	24.4	4.7	1.8	1.0	1.0	10.3	68.5	25.6	5.9	1.6	1.0	
		Post Chemo 1							8.2						1.3

Table 15.3 shows results obtained from flow cytometric analysis of tumour samples obtained by fine needle aspiration before, after and, in some cases, during chemotherapy.

Adr Mean = Ratio of mean red fluorescence of a sample taken one hour post adriamycin infusion to a sample taken just prior to treatment.

* = Histograms not interpretable.

Legend otherwise as for Table 15.1.

Figure 15.4 Fine Needle Aspirate Examined by Flourescence Microscopy

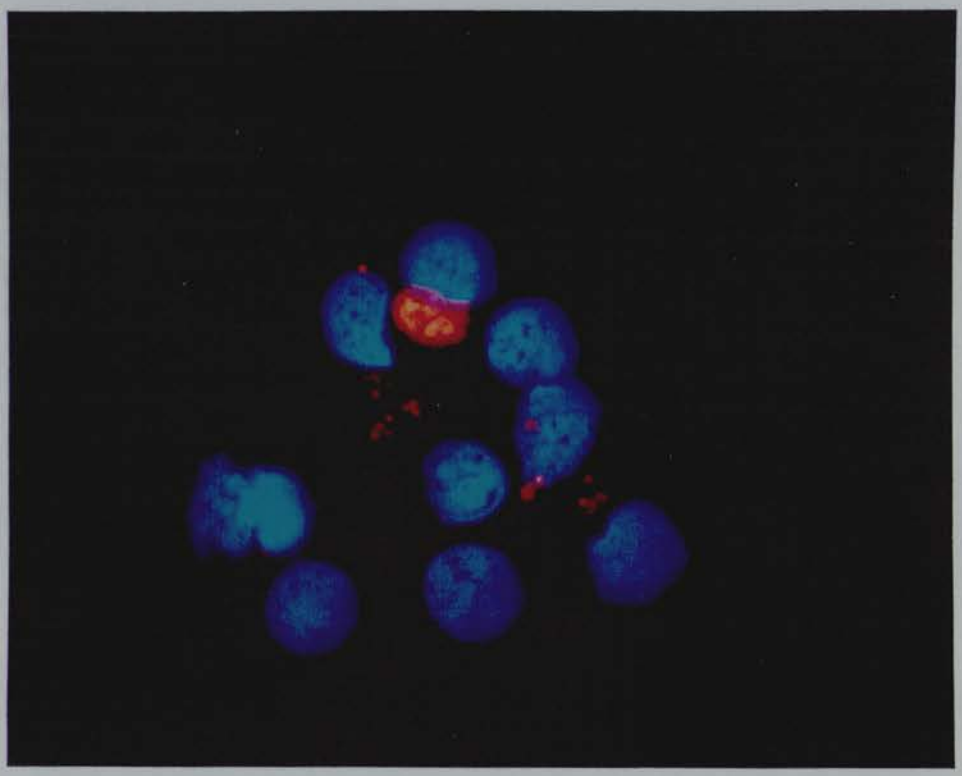


Figure 15.4 shows cellular material derived by fine needle aspiration of tumour 15 (see table 15.3) one hour after intra-venous infusion of adriamycin, and examined under a fluorescence microscope. Adriamycin within cells shows as a red colouration.

Figure 15.5 Measurement of Adriamycin Uptake by Flow Cytometry

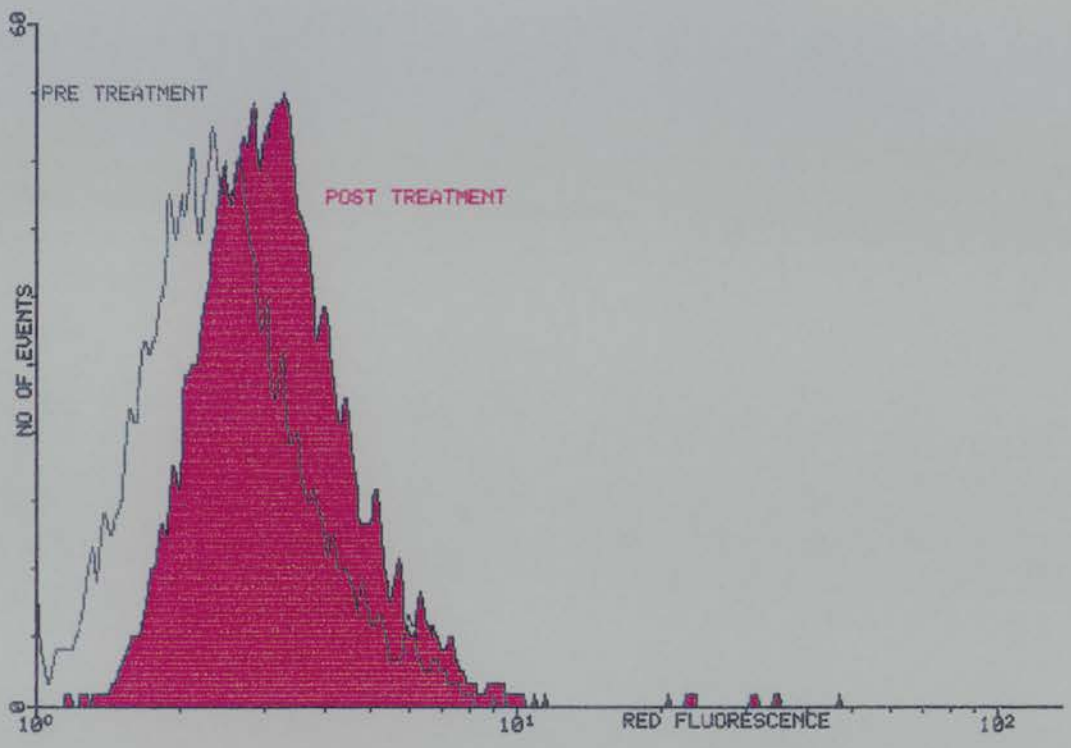


Figure 15.5 shows superimposed histograms created by the flow cytometer demonstrating red fluorescence in FNA samples taken from a breast cancer prior to and one hour after intravenous infusion of adriamycin. The mean increase in cellular fluoresence after treatment represents cellular uptake of adriamycin.

Figure 15.6 Series of DNA Histograms from Patient 20

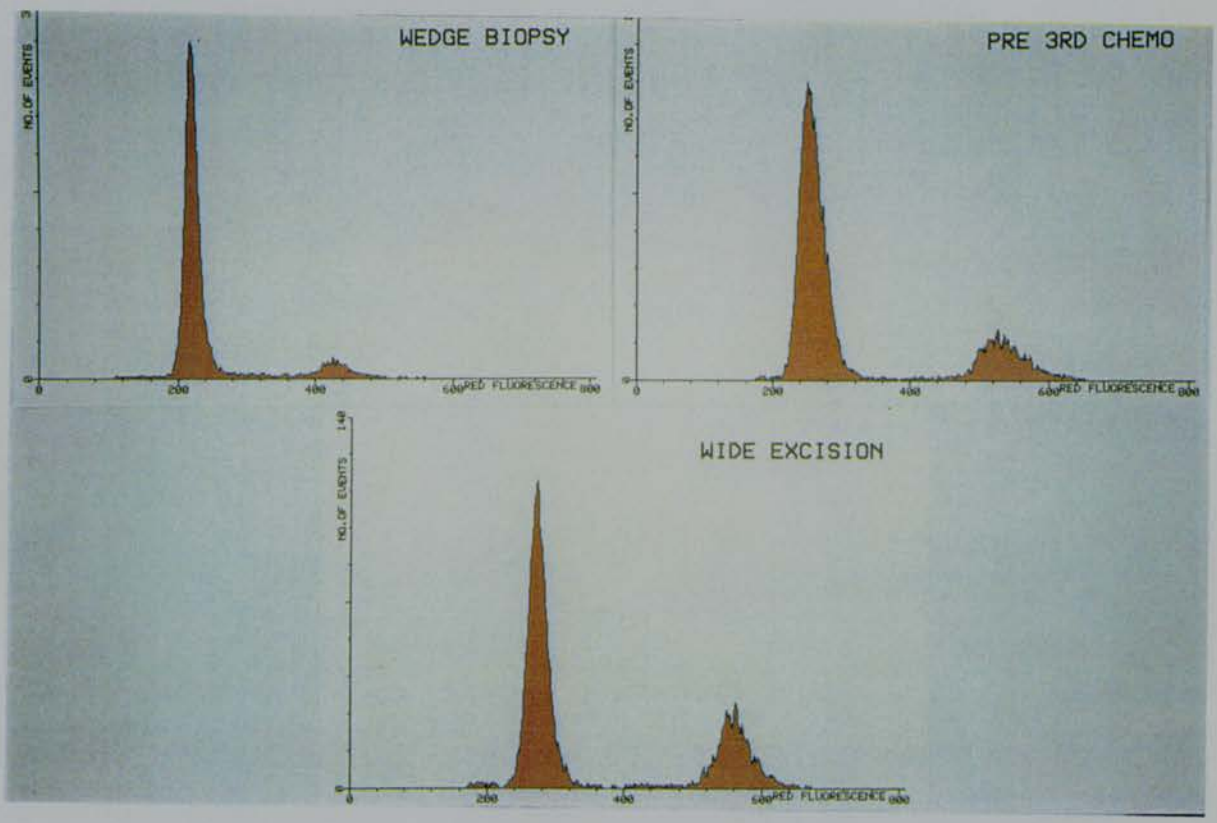


Figure 15.6 shows a series of histograms taken at a) time of wedge biopsy, b) prior to the third cycle of chemotherapy and c) at the time of definitive surgery. Note the accumulation of cells in the G_2/M phase of the cell cycle.

Figure 15.7 Series of DNA Histograms from Patient 18

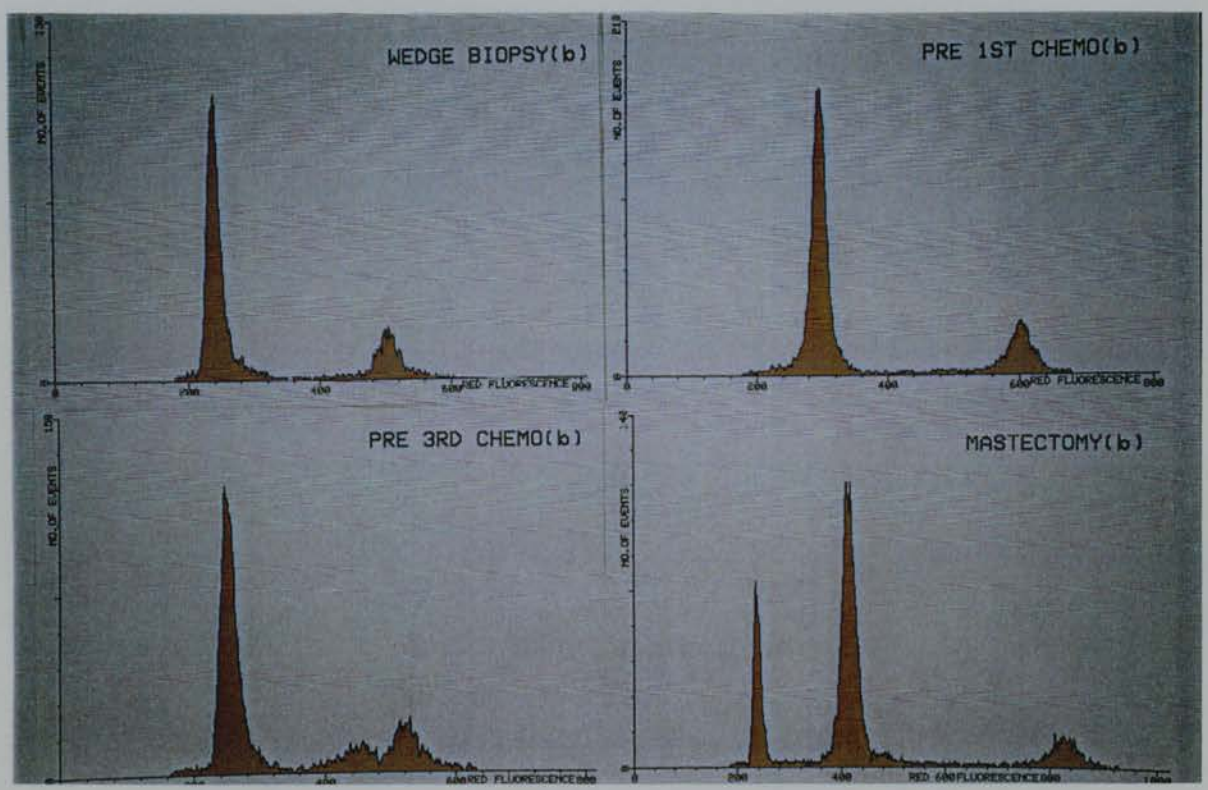


Figure 15.7 shows a series of histograms taken at a) time of wedge biopsy, b) prior to the first cycle of chemotherapy, c) prior to the third cycle of chemotherapy and d) at the time of definitive surgery. Note the appearance of an aneuploid subpopulation that becomes increasingly significant.

DISCUSSION

Introduction

This thesis aims to characterize the response of primary breast tumours to systemic therapy involving the use of both cytotoxic chemotherapy and anti-hormone agents. The following discussion will focus principally on tumours in elderly patients treated with tamoxifen simply because of the greater numbers of this particular population presenting to the Edinburgh Breast Unit during the study period. Firstly the clinical responses achieved in both the elderly patient and the primary systemic therapy trials will be discussed and thereafter the approach to, and the results of, the assessment of tumour biological responses to therapy will be reviewed. Finally, as this thesis covers a wide variety of aspects of response assessment, the significant findings will be discussed in terms of the prospects for further research.

Chapter 16 : The Clinical Response to Primary Systemic Therapy

16.1 : Tumour Measurement

Primary systemic therapy allows a direct assessment of the sensitivity of a particular tumour to a particular therapy by monitoring the tumour response *in situ*. Several different methods of monitoring response have been proposed ranging from serial tumour diameter measurements with engineers' callipers to Magnetic Resonance Imaging (Gilles, 1994). The Edinburgh Breast Unit has assessed the use of ultrasound imaging in measuring breast tumours and as a means of monitoring the response to treatment in an early series of patients treated in the primary systemic therapy trial (Forouhi, 1994). Ultrasound scanning is, to a certain extent, operator dependent and therefore it was necessary for the benefit of this thesis to confirm its accuracy in tumour measurement when operated by the author. A good correlation between ultrasound measured tumour diameter and that measured pathologically was obtained. A similarly good correlation was obtained between clinical diameter, measured with the use of engineers callipers, and pathological diameter . However, the correlation at smaller tumour volumes was poorer and indeed some tumours in the series that had been measured by ultrasound could not be clearly identified on clinical palpation and calliper measurements could not therefore be performed.

Forouhi et al. demonstrated that sequential calliper measurements were an accurate method of monitoring response to treatment. However the majority of patients in their study underwent primary chemotherapy with often very large reductions in tumour volume. The response to tamoxifen over the first three months of treatment is not usually of the same order. Calliper measurements are indirect in that the skin and subcutaneous tissues are included in the measurement. The thickness of these tissues can vary with time as can the degree of compression exerted through the callipers by the operator. The former problem has been addressed by the use of estimates of the thickness of the subcutaneous adipose tissue (Dixon, 1984b), but this is time-consuming and not always accurate. The latter problem is aided by employing a single operator to make all measurements. These potential inaccuracies may make comparisons of serial measurements on individual tumours difficult. Therefore, in order to measure what were likely to be small changes in tumour volume with tamoxifen therapy, the greater reproducible accuracy of ultrasound was thought to be preferable. An accurate assessment of response was particularly important as relationships with corresponding biological responses were to be examined. Ultrasound measurements were made on all tumours reported in this thesis but as a

comparison calliper measurements were taken where possible.

16.2 : The Response to Tamoxifen

There have been very few reports in the literature concerning the early response of breast tumours to tamoxifen therapy and none to our knowledge using ultrasound as a means of assessment. A previous study from Edinburgh examining predictive factors for long term "worthwhile" response to tamoxifen detailed response at three months in a group of unselected patients with both ER negative and positive tumours (Gaskell, 1992). Response was measured by engineers' callipers and graded according to U.I.C.C. criteria. Of 59 tumours assessed 12 (20%) demonstrated either a partial (11) or a complete (1) response, 35 were graded as static and 12 demonstrated progression. In the group of 95 patients reported in Chapter 14, 80 were assessed for U.I.C.C. response. In this population all tumours were ER positive and 27 (34%) demonstrated either a partial (19) or a complete (8) response, 51 were graded as static and only one tumour was classified as showing progression. The relatively high number of complete responses and low number of progressive tumours probably reflects the ER positivity of the group studied here. The U.I.C.C. criteria of response are based on clinical measurements with the attendant potential drawbacks outlined above and give little indication of the degree of response as a continuum for an individual tumour. By sequential ultrasound scans it has been possible to characterise the early macroscopic response to tamoxifen for the first time. Somewhat surprising was the rate at which tumours can respond. After only one month of therapy 44% of tumours had demonstrated a reduction in tumour volume of greater than 10%. By two months 64% had responded and after three months a total of 74% of tumours had responded. Alternatively of those which were going to respond by three months 61% and 87% had demonstrated a decrease in ultrasound assessed volume by one and two months respectively. A figure of 74% of tumours responding to tamoxifen is similar to that reported in the literature. It is a little higher than some reports but the 25 patients followed to six months of treatment may give a clue as to the reasoning behind this and the suitability of three months as a determinant for likelihood of response. Although only relatively few patients were studied beyond three months no patient classified as static demonstrated a response by six months and five patients who had demonstrated a response showed signs of tumour regrowth. It seems, therefore, that by ultrasound the vast majority of the tumours that are going to respond have been detected by three months of treatment. In addition some early responses may have been demonstrated in a group of tumours that develop resistance to tamoxifen by three to six months of

treatment and would have been classified in other studies, based on a later assessment, as non responders.

The degree of response observed at three months asks questions of the underlying histopathological processes. The responding tumours decreased by a mean of 58.5% in ultrasound measured volume. Of the 70 responding tumours 19 decreased by more than 75% in volume and of these two patients who underwent local excision after three months of treatment had achieved a complete pathological response. These observations prompted a detailed study of both cellular proliferation and, indirectly, cell death occurring in the cells of the tumours for which response had been assessed. The results of these studies are described in Chapter 15 and discussed below. The clinical implications of such responses with short term treatment are also important, particularly perhaps in the elderly population. It remains to be seen whether the degree of short term response predicts for overall recurrence-free survival in this population as the follow-up time is still relatively short. In attempting to determine predictive factors for overall worthwhile response to tamoxifen Gaskell *et al.* (Gaskell, 1992) failed to show a predictive value for response at three months as assessed by U.I.C.C. criteria or by sequential calliper measurements demonstrating a consistent trend of reduction in size over the three months. Horobin *et al.* (Horobin, 1991) showed that only 30% of those patients with ER positive tumours demonstrating a response to tamoxifen had persisting control of disease at five years. Thus it seems thst the majority of patients will require surgery at some point. If, however, surgery is delayed for three to six months the form of surgery required can be downstaged (i.e. from mastectomy to wide local excision) which is important in this population in that it is a shorter operation and can if necesary be performed under local anaesthetic and has lower attendant morbidity. Indeed in this study population of 26 patients who proceeded to local surgery and who were assessed prior to tamoxifen as requiring a mastectomy 10 patients were treated effectively by wide local excision.

The data generated from the study of the response of individual tumours to short-term tamoxifen therapy provided a powerful base on which to examine clinical and biological factors which may predict and/or reflect the observed responses. These are dicussed further below.

16.3 The Trial of Primary Systemic Therapy

The Edinburgh trial of primary systemic therapy for breast cancer commenced recruitment in January 1990 and had randomised 67 patients prior to the author's involvement. Over the time period of this present study a further 48 patients were entered into the trial, of whom 22 were randomised to receive primary systemic therapy and of these, two patients changed their minds after randomisation and opted for conventional management. This relatively small group of patients were further subdivided into treatment groups according to the oestrogen receptor status of their tumours (11 being ER negative and nine ER positive). The numbers were boosted slightly by the inclusion of five patients who expressed a wish to receive primary systemic therapy outwith the trial. These still, however, represent small numbers from which to draw any significant conclusions.

The move towards primary systemic or "neoadjuvant therapy" as a treatment option for primary breast cancer originated from the results of systemic therapy for locally advanced inoperable tumours. Overall response rates of 70 - 91% were reported for anthracycline containing regimens (Swain, 1987; Hortobagyi, 1988; Jacquillat, 1988; Mauriac, 1991). In these trials responses were determined clinically by calliper measurement and responders defined by U.I.C.C. criteria. These studies also demonstrated an increased survival in those patients responding well to primary therapy with, for example, one study demonstrating a clear survival advantage for those whose tumours had reduced in clinically estimated volume by greater than 75% in comparison with those with only a 50-75% reduction (Jacquillat, 1988). The trials of neoadjuvant therapy for early, operable, breast cancer are still reporting with relatively short median follow-up periods but the response rates to the initial chemotherapy are in keeping with the figures quoted in the earlier studies. The initial pilot study of primary systemic therapy in Edinburgh achieved a response rate of 85% with primary CHOP (cyclophosphamide, adriamycin vincristine and prednisolone) chemotherapy, if patients having failed initial endocrine therapy were excluded (Anderson, 1991). Bonadonna, one of the pioneers of primary systemic therapy, treated five groups of 33 women, all presenting with primary breast tumours greater than 3cm in clinical diameter, with five different chemotherapy regimens. There were no significant differences in response rates with different drug regimens with response rates of 70-87% reported (Bonadonna, 1990). In the study reported in this thesis, fifteen patients received primary therapy with cyclophosphamide, adriamycin and prednisolone and of these 14 (93%) demonstrated a response, all acheiving a greater than 50% reduction in tumour volume, as assessed by ultrasonography, with a mean

reduction in volume of 87%. The response rate obtained is high but given the small numbers is probably within the range that would be expected from the results of the reported studies. Furthermore ten patients (67%) responded with a greater than 75% reduction in volume and three (20%) achieved a complete histopathological response with no evidence of invasive carcinoma in the specimens obtained after treatment by mastectomy or wide local excision. Bonadonna's study suggested that at three years, relapse free survival was greater in those that had demonstrated the greater initial response to primary therapy (confirming Jacquillat's earlier findings in locally advanced disease). In his study seven of eight patients with a pathological response to primary therapy were disease free at three years. The 15 patients reported here have a median follow-up of 24 months (range 16 to 30 months) and of these five have developed metastatic disease and four have died of their disease. Included in the deaths was the one patient in the series who had not responded to chemotherapy but of the four other recurrences all had responded with a reduction in tumour volume of 88% or greater and, indeed, one had achieved a complete pathological response. It seems unlikely therefore in this series that the degree of response will be significantly related to survival. This group of tumours which have recurred early after an apparent good response to primary therapy are particularly important for further study. If indicators of poor prognosis and likely early recurrence could be identified either before treatment or derived from biological changes induced by treatment then these patients may be candidates for more intensive, high dose, chemotherapy. The two other recurrences in this present series (including one patient ultimately dying of her disease) ocurred in the only patients whose tumours were unresponsive to tamoxifen and which also, subsequently, proved unresponsive to chemotherapy. The observation that ER positive tumours which fail endocrine therapy tend to respond poorly to chemotherapy thereafter was also made by Anderson et al. in the earlier Edinburgh study. A possible explanation lies in the observation that *P* -glycoprotein expression (and therefore multi-drug resistance) appears to be induced, or selected for, during tamoxifen therapy in these tumours. This proposition has been raised by the investigation of tissue fom these and other tamoxifen resistant tumours during this study and will be discussed further below.

Nine patients with ER positive tumours were treated with tamoxifen with seven (78%) demonstrating a response. The responses were generally slower and of a smaller overall degree than those achieved by chemotherapy. The mean reduction in tumour volume over the three months of therapy was 70% with five tumours demonstrating reductions over 50% and a mean time to reach 50% of the initial volume of 38 days (in comparison with 27 days for those treated with chemotherapy).

The trials of primary systemic therapy reported to date have principally determined tumour response rates and thereafter whether a significant number of patients can avoid mastectomy and be safely treated by breast conservation. Results have been encouraging with figures for the number of patients avoiding mastectomy after treatment ranging from 20-88% (Bonadonna, 1990; Mauriac, 1991; Rodier, 1993; Smith, 1993). In addition all studies report low rates of local tumour recurrence after conservational surgery, in keeping with the experience when this procedure is employed as primary treatment (Fisher, 1989). The option to downgrade the surgical procedure required in individual cases has not, as yet, been included in the Edinburgh protocol, and all patients deemed to require mastectomy prior to primary systemic therapy have undergone this procedure after treatment regardless of response. It may well be that the downstaging of the local surgery required is the main benefit of primary as opposed to adjuvant therapy and this option should be included in any future trials.

Some of the theoretical arguments proposed to support the concept of primary systemic therapy would be expected, if borne out clinically, to lead to improved survival. Improved survival is also needed to justify treating smaller primary breast cancers with primary systemic therapy as, in this group, conservational surgery would be possible at presentation. The relatively few randomised trials testing primary therapy against conventional post-surgical adjuvant therapy. These trials are still ongoing and are yet to report. One small, non-randomised, French study has found no difference in relapse-free survival between the two treatment groups at a median follow-up period of 33 months (Rodier, 1993). A larger study, also non-randomised, with a similar median folow-up found more frequent isolated local recurrences but an improved overall survival in patients receiving primary systemic therapy (Mauriac, 1991). The group of 26 patients randomised to conventional therapy and reported here have now been followed-up for a median of 25 months. There have been four recurrences (all distant metastatic disease) and two deaths. At this early stage in follow-up and with these small numbers there appears to be little difference between the two trial groups in terms of survival. It is likely that national randomised trials will be required to determine survival benefits with any degree of significance. The NSABP (National Surgical Adjuvant Breast and Bowel Project) B-18 trial, instituted in the U.S.A. and Canada in 1988, with a projected accrual of 1275 patients, has as one of its main aims the determination of relapse-free and overall survival in patients treated with the same chemotherapy either pre- or post-operatively (Fisher, 1991b). The results of this trial are awaited with interest.

The clinical data generated by the present study comprising records of the rate and

degree of response of individual tumours to either chemotherapy or tamoxifen provided a base upon which to build the study of the underlying tumour biology. The main aims of such a study were to i) detect markers that would predict for response, preferably in material obtained by fine needle aspiration and ii) find markers which by their change of expression during treatment might reflect the sensitivity of the tumour to that particular treatment. This might also help in predicting those patients likely to relapse early and who may, therefore, need additional or more intensive therapy. Such a study should also generate data that provide insights into mechanisms of action of, and resistance to, individual therapies. The results of these investigations are discussed in the following chapter.

Chapter 17 : The Biological Response to Primary Systemic Therapy

17.1 Cell line Experiments

The broad approach to the investigation of cellular characteristics which may predict for or reflect tumour response to systemic therapy involved the development of methodology in model systems before the application of techniques to clinical material. Such model systems comprised either tumour derived cell lines grown by tissue culture techniques or as xenografts in immunocompromised animals and a primary breast tumour grown as a xenograft.

Several studies have linked the rate of tumour proliferation to the response observed with systemic therapy. Tumours with high proliferative rates tend to have a greater response to chemotherapeutic agents (Remvikos, 1993b; Spyratos, 1992) and possibly also to hormonal manipulation with agents such as tamoxifen (Paradiso, 1988) although this is more debateable. However it would be expected that for both forms of systemic therapy a change in tumour cell proliferation would accompany response. Therefore, it was felt to be important to make an assessment of proliferation as both a predictor and reflector of tumour response. Three methods of measuring tumour proliferation were tested.i) The classical flow cytometric "S -phase" measurement derived from cell cycle analysis of cells stained for their DNA content, ii) the incorporation of the thymidine analogue, bromodeoxyuridine and iii) the expression of cell cycle-related nuclear antigens, Ki-67 and Ki-S1. All methods involved the use of the flow cytometer as the aim was to develop methodology applicable to material obtained by fine needle aspiration of breast tumours. In cell lines the three methods correlated well with each other, the closest relationship being that between S-phase and BrdU uptake. After these initial experiments no further work was carried out with Ki-67 because of the poor stability of the antigen on freezing which would have made it unsuitable for the examination of stored clinical samples.

To assess the suitability of these techniques to material derived from fine needle aspirates from primary breast tumours the three markers were assessed in a group of unselected patients. As in the cell line experiments, significant correlations were observed between the markers although the degrees of association were lower in the clinical samples. This may be because the material derived from a fine needle aspirate of a breast tumour is heterogenous comprising several cell types whereas that from a cell culture should be homogenous. This factor may be expected to have implications for the measurement of each of the parameters because there is no certain way of distinguishing benign, stromal cells from the malignant cells by flow cytometry. The

identification of malignant cells is possible indirectly if by their DNA content they are defined as being other than diploid. This is illustrated by the good correlation obtained between S-phase fraction and BrdU uptake in aneuploid tumour cells and the lack of correlation between these two parameters in diploid tumours. Other possible reasons underlying the poor correlation between S-phase and BrdU uptake relate to the *in vitro* method of measuring the latter parameter. The cell line experiments involved a simple trypsinisation step to remove cells from the plastic culture bottles and thereafter incubation in BrdU containing media. The tumours from which the fine needle aspirates were taken had all been surgically resected and had therefore suffered a degree of ischaemia despite the samples being taken as soon after surgical removal as possible. The mechanical process of needle aspiration involves removing cells from their environment and also must induce a degree of damage both of which may limit the capability of the cells to synthesise DNA. This is compatible with other reports in which the *in vitro* technique of BrdU incorporation has been used (Remvikos, 1991; Buley, 1992).

These techniques were then applied to xenograft models in an attempt to monitor tumour proliferation during systemic therapy in material derived from serial fine needle aspirates. This is the first report of such investigations. The use of the model system had the advantage that tumour growth and proliferation could also be studied in control tumours not undergoing systemic therapy (which is not usually ethically feasible in human primary tumours). Additionally, frequent sequential samples could be taken and assessed. The two xenografts studied in detail, ZR 75.1 and T1068 were of differing origins and displayed different growth characteristics. The former, an established ER positive breast cancer cell line which had then been successfully grown as a xenograft, grew relatively slowly with only a moderately high flow cytometric S-phase fraction. T1068 xenografts were derived directly from a primary mucoid breast carcinoma and grew rapidly with a very high S-phase fraction.

ZR 75.1 control tumours, monitored during the experiment described in Chapter 12, continued to grow at a steady rate with an increasing S-phase fraction as measured by flow cytometric analysis of specimens obtained by fine needle aspiration. The treated tumours responded well to both 2.5 and 5mg of implanted tamoxifen with a decrease in the two parameters measured to assess cellular proliferation by two weeks after the commencement of therapy. Another change noted in the serial samples taken for cell cycle analysis was an increase in the proportion of cells within the G_0/G_1 phase of the cell cycle in treated, but not control tumours, in the sample taken two weeks after the commencement of therapy. This confirmed earlier reports on the effects of tamoxifen on the cell cycle (Sutherland, 1983; Osborne, 1983), although the majority of previous

work has been performed in tissue culture systems. The decrease in tumour size lagged the change in cellular proliferation and cell cycle distribution by approximately one week. This may have implications in the clinical setting in that it is important to find indicators of tumour response as early in treatment as possible so that alternative therapies may be substituted in resistant tumours. If a decreases in S-phase fraction or an increase G_0/G_1 phase fraction occur early in the treatment of human tumours these may be valuable predictors of response and in addition could potentially be measured on material obtained by fine needle aspiration. The other means of assessment of cell proliferation, BrdU uptake, measured in a subgroup of tumours, showed slightly different changes. All tumours showed an increase in BrdU uptake at two weeks compared with that measured in the pre-treatment samples, although the greatest increase was in the control samples. All tumours thereafter showed a decrease in uptake, controls as well as treated tumours. These findings are not easily explained and needs further investigation with larger numbers before meaningful conclusions can be drawn.

The T1068 xenograft, however, behaved differently in that control tumours showed a steady decline in cellular proliferation with increasing tumour size, with a good correlation between the two parameters of proliferation measured. This might be explained by the fast rate of growth displayed by these tumours so that they may have outgrown their blood supply early in the experiment and hence an increasingly smaller proportion of tumour cells within a given aspirate would show evidence of DNA accumulation. This explanation is supported by the observation that one tumour measured to be very small at the start of the experiment showed a consistently high S-phase fraction throughout the experiment, despite systemic treatment (Tumour 2b table 11.3). The T1068 xenograft demonstrated significant resistance to increasing doses of adriamycin therapy. Although there was a tendency for a progressively lower rate of tumour growth with increasing doses these differences were not significant. All tumours treated with 0.5mg/kg adriamycin showed a decline in S-phase fraction and BrdU uptake similar to that seen in the control tumours. However, in this group of tumours, unlike the controls, there was not a good correlation between the values of these two parameters in individual tumours. There was the impression, given the limitations of small numbers, that serial BrdU uptake measurements were a more accurate method of assessing proliferation since serial values showed a more consistent decline than the more scattered values for S-phase. A potential complication in measuring S-phase in adriamycin-treated tumours is that the drug acts upon cells synthesising DNA, classically causing an accumulation of cells within late S / G_2M phase. No such increase in cells within the G_2M phase could be demonstrated in the

experiment reported above in which the tumours appeared particularly resistant to adriamycin. An alternative explanation may be related to the fact that adriamycin displays intrinsic fluorescence within the same part of the spectrum as propidium iodide, used to stain DNA for cell cycle analysis. Adriamycin bound to the DNA, in the treated group, may therefore interfere with the analyses.

In summary, the series of experiments with xenografts provided insight into tumour growth kinetics by employing the techniques of serial fine needle aspiration and flow cytometry. They illustrated the potential value of cell cycle analysis with measurement of S-phase and G_0/G_1 phase in the assessment of early response to tamoxifen but also the potential problems of such measurements in adriamycin therapy. It was felt that in the assessment of human primary tumours undergoing systemic therapy the technique of serial fine needle aspiration should be tested and that at least two methods of assessment of celullar proliferation should be used.

17.2 Clinical Material - Tamoxifen Therapy

Tumour samples were obtained from 53 patients before and after a median of 18 weeks treatment with tamoxifen. To date there are very few studies that have reported results using such material and none that have accurate clinical response data for the tumours studied. Each of the markers detected by immunohistochemical staining in this group of tumours will be discussed in turn but firstly reference will be made to the measurement of oestrogen receptors and the response to tamoxifen.

a) Oestrogen Receptors

50 tumours had pre-treatment ER measured both by EIA, on material obtained by wedge biopsy, and ER-ICA, on cells obtained by fine needle aspiration. Such samples were valuable in assessing the predictive value for response of these respective methods of measurement. All tumours studied were deemed ER+ve by the fact that they contained receptors at >20 fmol/mg cytosol protein by EIA, although three tumours did have a value of <10% cells staining by ER-ICA. Thus, unlike previous studies, where ER positivity as assessed qualitatively by either method has shown to be a good predictor of overall response to endocrine therapy (****)(Skoog, 1991) this study was concerned with the relationship between quantitative values of both ER expression and tumour response. Values obtained by both methods of analysis significantly correlated with the actual tumour volume reduction as assessed by ultrasound over the relatively short treatment period. EIA produced values for ER that correlated with response to a higher degree of significance than ER-ICA and gave a cut-off value of 222fmol/mg, levels above which always related to a response during the treatment period. No such "cut-off" value existed for ER-ICA estimations. In a slightly different population of 53 patients, studied for changes in expression of other markers before an after treatment, the ER value as assessed by EIA was the only marker which predicted for both overall and actual response in tissue obtained prior to treatment on multivariate analysis.

If primary systemic therapy for a short period is to be used primarily to achieve such a degree of tumour shrinkage as to avoid mastectomy then these results become highly significant. This is particularly pertinent in the elderly population in whom delay of surgery should be avoided unless there is a high likelihood of response to tamoxifen therapy. It appears from these results that EIA estimations of pre-treatment ER content are particularly useful in the prediction of the quantitative response to tamoxifen. Unfortunately such analyses are dependant upon a requirement for biopsy material

and, where this is not available or appropriate, ER-ICA estimations are still predictive of response although not with such accuracy as EIA.

b) pS2

Expression of the oestrogen-inducible pS2 gene has, not surprisingly, been shown to occur predominantly in ER+ve breast tumours (Rio, 1987) and has been suggested to be an indicator of an intact ER mechanism, in similar fashion to progesterone receptor expression. In the group of exclusively ER+ve tumours in this study there was, however, no correlation between the degree of ER expression and the pS2 staining index in material taken prior to tamoxifen. This confirms previous studies in breast tumours of both pS2 protein expression and mRNA content which found a similar poor quantitative correlation with ER (Manning, 1993; Luqmani, 1993). Interestingly there was a positive quantative correlation between the expression of pS2 after tamoxifen therapy and ER content prior to treatment. This could be interpreted as displaying evidence for oestrogenic effects of tamoxifen with greater induction of pS2 in tumours expressing higher levels of ER. This relationship appeared independent of tumour response as statistical significance was lost if only responding tumours were analysed.

The expression of pS2 has been suggested to be a marker of responsiveness to endocrine therapy, and has even been suggested, in some studies, to be a better predictor of response than ER status (Schwartz, 1991). Most studies however have found no advantage for pS2 over ER status as a predictor of response (Skilton, 1989; Luqmani, 1993). This study found no predictive value for pS2 protein expression, as assessed by immunohistochemistry, for either the overall or the degree of tumour response in this selected group of ER+ve tumours. There was a significant trend towards higher levels of pS2 expression after treatment in tumours that had demonstrated a response than those which had not. This finding has been suggested by one previous study (Henry, 1989). Although this inferred increase in pS2 expression in responding tumours was clear on comparison of pre- and post-treatment samples of individual tumours the trend failed to reach statistical significance.

It had been hoped that pS2 would provide an additional tool to ER to improve the selection of patients likely to respond to tamoxifen. To this end during the course of this study a new technique to measure pS2 expression by flow cytometry was developed, initially in tissue culture systems. This was then applied to cellular material derived from FNA's of primary breast tumours. With FNA samples allowing assessment of potential changes in antigen expression early in treatment an increase in

pS2 may be of prognostic significance both for short and long-term response. The small series of patients reported who underwent serial FNA's did show increased levels of pS2 at 6-8 weeks of treatment. However this did not correlate with response and, interestingly, several non-responding tumours displayed, in comparison to pre-treatment levels, an initial increase in pS2 before a decrease at the time of definitive surgery.

In summary it appears that pS2 protein expression measured either by immunohistochemistry on tumour tissue samples or by flow cytometry of material derived from FNA's can provide little extra value in determining short term response to tamoxifen. The relationship to long term response and relapse-free survival will be interesting to follow and further work on serial sampling of tumours undergoing treatment may aid understanding of tamoxifen interactions with ER mechanisms within the cell.

c) EGF-R

The expression of the EGF-Receptor in breast tumours has been described as an indicator of poor responsiveness to tamoxifen therapy (Nicholson, 1993). However, many studies have described an inverse relationship between EGF-R expression and ER content (Sainsbury, 1985; Bolla, 1992; Bilous, 1992). It may be, therefore, that the relationship merely reflects the ER status of the tumours with ER-ve/EGF-R+ve tumours showing low rates of response to tamoxifen. The group of tumours in elderly patients, reported in this thesis, were all ER+ve and, as expected, there was a low level of EGF-R expression. Interestingly, however, among the EGF-R+ve tumours there was a significant positive correlation between quantitative measures of expression and response. This is not easy to explain, and indeed it may be dangerous to attempt such given the small numbers but a putative explanation may lie in the functions of the EGF-R. It is thought that one of the most important ligands of the receptor is the growth factor, TGFα, which shares approximately 35% homology with epidermal growth factor (Derynk, 1986). TGFα induces cell proliferation and is also thought to be important in tumour angiogenesis (Jordan, 1993). Tamoxifen has been shown to decrease levels of TGFα and this has been proposed as a possible mechanism of anti-tumour action (Noguchi, 1993). Tumours with greater levels of EGF-R and with therefore more dependance on stimulatory factors such as TGFα may respond to a greater degree than those without. It will be interesting to follow this

initial observation in greater numbers of tumours.

d) c-*erb*B-2

Positive membrane staining for the c-*erb*B-2 protein product was detected in 27% of tumours prior to therapy which is in keeping with figures of 14 to 42% reported in previous series of breast tumours assessed by immunohistochemstry (Gusterson, 1988; Nicholson, 1993; Lipponen, 1993a). These studies, unlike that reported here, did not take oestrogen receptor status into account and, indeed, a negative correlation has been suggested between the two markers (Gusterson, 1992; Todd, 1992). One might expect, therefore, a lower proportion of positively staining tumours in an exclusively ER+ve series, although other studies have shown gene amplification or immunohistochemical staining in 9.7-30% of ER+ve tumours (Todd, 1992; Skoog, 1992).

In the group of 51 tumours reported here there was no apparent relationship between c-*erb*B-2 expression prior to treatment and the observed response to tamoxifen after three months. In cell lines, transfection of the c-*erb*B-2 gene has been shown to confer tamoxifen resistance *in vitro* and *in vivo* (Benz, 1992) and there has been a suggestion that expession of the protein is also related to poor clinical response to tamoxifen (Wright, 1992; Nicholson, 1993). These studies, however, failed to also examine for ER expression and can therefore not be compared to that reported here.

There has been little previous work on the effect of tamoxifen on the cellular expression of c-*erb*B-2. One study of ER+ve and ER-ve breast cancer cell lines found induction of c-*erb*B-2 in the ER+ve cell line in keeping with an anti-oestrogenic effect (Antoniotti, 1992) (oestrogens, in previous studies, having been found to inhibit expression (Dati, 1990)). In clinical material, however, both a reduction and an increase in c-*erb*B-2 RNA levels have been shown with tamoxifen treatment in seperate series (Le Roy, 1991)[Johnson ***(refereed paper). Interestingly, in the former series, the effect was noted principally in ER -ve tumours. In the group of 51 tumours reported here no statistically significant trends in the change of expression were noted although the numbers of "positive" tumours were small. There was, however, a tendancy for an increase in staining in responding tumours and a decrease in staining in non-responding tumours. This will be intersting to review in larger numbers of tumours as induction/suppression of c-*erb*B-2 may, respectively, reflect intact or abnormal ER apparatus , and hormone responsiveness. The implications of short-term changes in expression for the long-term response to tamoxifen remains to be seen.

e) *P* -Glycoprotein

In the group of elderly patients studied the majority of pre-treatment biopsies were negative for staining with the polyclonal antibody to *P* -glycoprotein and although the proportion of positive samples was greater in patients not subsequently responding to tamoxifen the difference in relation to responding patients was not statistically significant. However there were highly significant differences in the percentage of tumour cells staining post-treatment with higher levels of staining in non-responding tumours than those which had demonstrated a response. Indeed in the multivariate analysis performed for all the markers studied *P* -glycoprotein staining after treatment was the only marker to have a significant relationship with clinical response other than ER expression prior to treatment. As implied by this finding there was indeed a significant trend towards increased *P* -glycoprotein expression in "non-responders" compared with "responders"

At this stage it is not possible to define the mechanism behind these observed changes with tamoxifen or even to determine whether increased staining seen in non-responders results from induction of *P* -glycoprotein or clonal selection of cells expressing *P* -glycoprotein. Several studies have demonstrated an interaction of tamoxifen with *P* -glycoprotein showing that high concentrations of tamoxifen inhibit *P* -glycoprotein-mediated resistance to chemotherapeutic agents *in vitro* (Ramu, 1984). Although such an effect has not been convincingly demonstrated clinically (Stuart, 1992) it seems likely that tamoxifen may be a substrate for the *P* -glycoprotein mechanism. This would be consistent with the speculation of the presence of an efflux pump mechanism invoked to explain the finding of low levels of tamoxifen and its metabolites in resistant tumours (Johnston, 1993). It is important to note, however, that a study of a MCF-7 breast cancer cell line transfected with the *mdr*-1 gene could not demonstrate resistance to tamoxifen (Clark, 1992). Equally not all the non-responders in this present study expressed *P* -glycoprotein and other mechanisms of resistance must be operative. However, clinical studies have shown *mdr*-1 gene amplification during tamoxifen therapy (Lonn, 1992) although this has subsequently been shown to occur during other endocrine therapies more frequently (Lonn, 1993). Nonetheless induction and/or clonal selection of *P*-glycoprotein expression may help to explain the relatively poor survival following chemotherapy of patients who have not responded to primary tamoxifen therapy (Swenerton, 1979). Additionally, early reports from the Edinburgh trial of primary systemic therapy noted a poor response rate to chemotherapy in ER+ve tumours which had not previously responded to tamoxifen (Anderson, 1991). Although only two patients failed tamoxifen therapy, in

the series from the trial reported in this thesis, both of these also appeared resistant to chemotherapy. Importantly their tumours were found to have increased expression of *P* -glycoprotein after tamoxifen, and prior to chemotherapy, as assessed by flow cytometry.

The relationship between *P* -glycoprotein expression and resistance to tamoxifen was unexpected, but nevertheless deserves further investigation since the implications both for combined hormonal and chemotherapeutic approaches to systemic treatment and the increasing use of adjuvant and prophylactic tamoxifen therapy are potentially very important.

f) p53

The low incidence of positive staining for p53 protein (11%) in the study group of patients treated with primary tamoxifen is not unexpected. Previous studies have shown a negative relationship between p53 expression and the presence of oestrogen receptors with figures ranging from 14 to 32% positivity in ER+ve breast tumours (Bosari, 1992; Poller, 1992; Friedrichs, 1993). One of these studies also demonstrated a significantly lower incidence of p53 expression in post-menopausal as compared to pre-menopausal patients. The group of post-menopausal patients with ER+ve tumours reported here confirm, therefore, the predicted low incidence of p53 expression. With such a low incidence of positivity it has been impossible to draw any meaningful conclusions from the data reported here. This is unfortunate, particularly in the light of the positive findings reported above which relate proliferative and surrogate apoptotic markers to tamoxifen response. Previously p53 mutations have been related to highly proliferative tumours (Allred, 1993), EGFR expression (Poller, 1992) and potentially therefore resistance to tamoxifen due to development of oestrogen independent growth promotion. Control of tumour cell growth has also been suggested to involve links between p53, *bcl* - 2 and c-*myc* expression and thus further work with these markers in larger numbers of patients would be interesting.

g) Ki-S1 and Bcl-2

Most early studies of the mechanism of action of tamoxifen suggested it to be a cytostatic agent (Sutherland, 1983). However, as demonstrated in the work reported in this thesis, patients often achieve dramatic responses to therapy with large reductions

in tumour volume. This may occur as a result of the normal process of cell death (which has been described in normal breast tissue (Ferguson, 1981)) being superimposed upon tamoxifen induced cytostasis. However tamoxifen might also directly increase rates of "programmed cell death"; the tamoxifen analogue toremifene having been shown to induce apoptosis in normal breast tissue by an, as yet, unidentified pathway (Warri, 1993). The present study included an examination of the events following tamoxifen treatment by measuring markers which may be associated with proliferation and apoptosis. Most importantly, it was also possible to make correlations with changes in the size of individual tumours assessed by ultrasound during the course of therapy thus providing a more detailed evaluation of clinical response than has previously been reported.

The incidence and degree of immunohistochemical staining for Ki-S1 were comparable with those reported by others for similar proliferation markers in breast cancer such as PCNA (Aaltomaa, 1992b) and Ki-67 (Nicholson, 1993). Similarly the incidence of positivity for Bcl-2 of 86% is compatible with the findings of Leek *et al.* (Leek, 1994) and Nathan *et al.* (Nathan, 1994), although somewhat higher than that reported by Johnston *et al.* (Johnson, 1994). However it is important to note that the present study was performed on oestrogen receptor-positive tumours in which the incidence of Bcl-2 has been reported to be higher (Chan, 1993; Leek, 1994).

The presence and degree of expression of Ki-S1 in pre-treatment biopsies did not predict for subsequent response to tamoxifen. Previous reports, in which markers of tumour cell proliferation such as S-phase and thymidine labelling index have been studied have found varying relationships between response and proliferation (Paradiso, 1988; Amadori, 1993). Although others have suggested that the presence of Bcl-2 in breast carcinomas is a better predictor of response to tamoxifen therapy than are oestrogen receptors (Gee, 1994), we were unable to show that simple presence of Bcl-2 was predictive for overall response to tamoxifen in the series of oestrogen receptor-positive tumours studied. However, there was a significant correlation between levels of pre-treatment Bcl-2 and the degree of subsequent clinical response to treatment. This result is superficially paradoxical since it might be expected that, if Bcl-2 is a marker of cell survival then high expression would be associated with a lesser degree of response to treatment. However, there are several potential explanations for this finding. For example Bcl-2 may be a surrogate marker for oestrogen responsiveness, either because of its relationship with oestrogen receptor (a significant correlation between levels of ER and Bcl-2 expression was demonstrated in pre-treatment biopsies) or because since oestrogen induces Bcl-2 in breast cancer cells (demonstrated *in vitro* (Gee, 1994)) its presence may reflect a state of oestrogen

stimulation. In either event cells staining positively for Bcl-2 may be more prone to antioestrogen effects.

The important feature of the present study is the ability to monitor changes in Ki-S1 and Bcl-2 with treatment and compare these with clinical response. Thus it was possible to show that, in keeping with tamoxifen's anti-proliferative effects, the majority of responding tumours showed a decrease in the expression of Ki-S1; similar effects using other markers have been reported by others (Clarke, 1993; Johnson, 1994). In contrast, the majority of non-responding tumours either showed an increase or no change in proliferation as monitored by Ki-S1. It should be noted, however, that whilst these group differences are highly significant there were substantial minorities in both responding and non-responding tumours which failed to show the appropriate changes with treatment. Thus, despite showing a clear decrease in tumour size, three tumours showed an increase in proliferation with tamoxifen treatment. It is possible that this reflects clonal selection of tumour cells which are both highly proliferative and resistant to tamoxifen. It will be of interest to determine whether with further follow-up these particular patients present with early recurrent disease. Conversely, within the group of 14 non-responding patients there were five tumours which showed a decrease in proliferation which was not translated into a reduction in tumour size. It could be that in these patients sequential measurements of tumour size does not accurately reflect cellular responses but equally the possibility needs to be addressed that proliferation is not the single or major mechanism by which tamoxifen exerts its effect on tumour growth.

In this respect it is widely accepted that tumour growth reflects a balance between proliferation and cell loss (in particular that occurring by apoptosis). It was because of this that measurements of Bcl-2 were included in the present study. If Bcl-2 is a marker of resistance to cell death then a reduction in the expression of Bcl-2 with tamoxifen treatment might be expected to be associated with response. Some experimental data support this in that exposure of breast cancer cells to antioestrogens appears to be associated with a decrease in Bcl-2 expression (Gee, 1994). Paradoxically, a recent small study (Johnson, 1994) has reported greater incidence of Bcl-2 expression in tumour samples taken after tamoxifen treatment, but these results were not related to response to treatment. The results of the present study are therefore particularly informative. These show that 21 of the 37 tumours responding to tamoxifen showed a decrease in the percentage of cell staining for Bcl-2. In contrast in non-responding tumours only a minority showed a decrease in expression. Whilst this is apparently at odds with the study of Johnstone *et al.* (Johnson, 1994), it should be noted that even the present study did identify individual tumours in which response to

tamoxifen was associated with an increase in expression of Bcl-2. However, the fall in Bcl-2 expression in tumours decreasing in size is consistent with the putative role of the Bcl-2 product as a factor influencing cell viability.

Given that decreases in Ki-S1 and Bcl-2 were both associated with response to tamoxifen it is important to determine whether these are a manifestation of a single process or whether they reflect independent mechanisms. It has been suggested that as tamoxifen induces cytostasis in G_1, and Bcl-2 has been shown to be largely absent during this phase of the cell cycle, there may well be a relationship between these two markers in tumours undergoing treatment (Gee, 1994). However, the observations reported here do not confirm this hypothesis. Thus, in only 11 of 51 tumours was the pattern of change with tamoxifen treatment similar for both markers and in nine of these cases this was to show concordant decreases in responding tumours. Furthermore, in terms of the quantitative changes which occurred with treatment combined assessment of both markers was significantly more strongly associated with reduction in tumour size than either Ki-S1 or Bcl-2 alone. It would seem, therefore, that tamoxifen treatment predominantly affected either Ki-S1 or Bcl-2 expression, suggesting a dissociation between mechanisms of cell survival and proliferation.

Whilst these results have illustrated profound effects of tamoxifen on the expression of markers associated with either proliferation or apoptosis and shown that these may differ in responding and non-responding tumours, the clinical utility of the findings is not immediate. Pre-treatment expression of the markers cannot discriminate between responding and non-responding tumours on an individual basis. Similarly, whilst changes in levels of expression are significantly different between responding and non-responding tumours, there are a substantial number which behave exceptionally. Response to tamoxifen in terms of effects on cellular proliferation and apoptosis clearly remains a complex issue but one that needs to be resolved if this agent and future antioestrogens are to be used optimally in breast cancer treatment.

17.3 Clinical Material - LHRH Agonist Therapy

Only four premenopausal patients with ER+ve tumours were treated with the LHRH agonist goserelin. The data generated from such a small population cannot be used to draw any significant conclusions. However, it was interesting to note that all tumours responded to treatment and all demonstrated a decrease in pS2 staining. This would be indicative of pure antioestrogenic effects (indirectly via the inhibition of oestrogen production) as opposed to the trend towards induction of pS2 demonstrated in tamoxifen treated tumours. The observation that two of the four tumours showed increased staining for *P* -glycoprotein after goserelin therapy whilst a third had high levels both before and after treatment deserves further attention. It would be interesting and important to examine a larger number of patients given primary treatment with LHRH agonists and review the response to second line treatment with chemotherapy in this group. Mixed changes in staining were observed for c-*erb*B-2, Bcl-2 and Ki-S1.

17.4 Clinical Material - Chemotherapy

Only limited conclusions may be drawn from material derived from patients treated by chemotherapy as per the protocol for the trial of primary systemic therapy. This is primarily due to the low number of patients recruited during the study period and to a lesser extent a result of the small volume and poor quality of material available from tumours after treatment. Many tumours displayed a 90% or greater reduction in volume and some showed a complete pathological response. In addition only one tumour failed to respond to chemotherapy, therefore it is dificult to make comparisons between response and immunohistochemical staining. Perhaps most interesting are the staining patterns with Ki-S1, c-*erb*B-2 and *P*- glycoprotein antibodies. The only tumour to show an increase in proliferation during treatment (as defined by an increase in Ki-S1 staining) was that which failed to respond.

The non-responding tumour would perhaps be expected to show an increase in proliferation and it was reassuring that no responding tumours displayed such an increase. Indeed the one non-responding patient has since died during follow-up (after 16 months). However, the tumour material from this patient did not show positive staining for c-*erb*B-2. Positive staining with this antibody does not appear to be associated with poor response of the primary tumour to chemotherapy but yet in this group is a marker of poor overall prognosis, in that the two patients whose tumours stained strongly positive for c-*erb*B-2 have died of metastatic disease during the relatively short follow-up period. The implication of previous studies demonstrating the poor clinical outcome of patients receiving postoperative adjuvant chemotherapy for tumours overexpressing c-*erb*B-2 has been that the gene confers a degree of chemoresistance (Gusterson, 1992; Allred, 1992b). If this is the case such resistance was not assessable, in this present study, by measuring the reduction in tumour volume with primary chemotherapy. The conclusion from this limited data is that c-*erb*B-2 expression does not predict for the initial tumour response, but, in fact, may a better marker for early relapse and poor survival following primary therapy than the degree of the observed clinical response. Of course the only patient not to respond to primary therapy has also died and overall response is likely also to be a predictor of survival.

P- glycoprotein expression, as has been described above, occurs in association with resistance to adriamycin chemotherapy. In this very small study population, only four tumours expressed *P*- glycoprotein prior to treatment, allbeit at low levels. This subgroup included the one non-responding tumour. Of the three that did demonstrate a clinical response two patients have since died and the remaining tumour showed the

smallest reduction in volume of the population studied. One of the tumours, despite demonstrating a 95% reduction in tumour volume, showed a marked increase in the percentage of cells staining for *P*- glycoprotein when post treatment tissue was compared to that taken prior to therapy. This would be in keeping with the clonal selection of resistant cells and this patient has since died with no response of recurrent disease to second line chemotherapy. An increase in staining index was seen in one additional tumour which had no significant staining prior to treatment. Although the staining index of the post-treatment specimen was relatively low it is possible that a clone of resistant cells remained after surgery and on clinical follow-up this patient should be monitored closely. The possible importance of *P*- glycoprotein expression in these patients clearly supposes that micrometastatic disease responds to therapy in the same fashion as the primary tumour. Further insight into this selection process may be gained from the flow cytometric assessment of serial fine needle aspirate specimens discussed below.

17.5 Clinical Material - Flow Cytometry and Fine Needle Aspirates

The tumour expression of several potentially interesting antigens have been shown by immunohistochemical techniques, as described above, to be related to the clinical response (and possibly survival) of patients treated with primary endocrine and chemotherapy. Fine needle aspiration of breast tumour xenografts and flow cytometric analyses of the material obtained also proved a useful technique in assessing tumour growth and response principally in terms of cell cycle analysis and other measurements of proliferation. This technique could also provide information as to the expression of other antigens removing the necessity for biopsies to obtain tissue fragments.

Flow cytometric assessment of material derived from fine needle aspirates produced differerent and some additional data when compared to those obtained by imunohistochemical investigations of tissue samples from the same tumours.

There was a clear trend towards response in aneuploid, and non-response in diploid tumours. This, however, did not reach statistical significance, in agreement with previous inconclusive studies (Robertson, 1991; Skoog, 1991). A possible explanation for this apparent trend would be related to the greater response seen in tumours with higher proliferative rates as shown in at least one previous study (Paradiso, 1988). Aneuploid tumours have been reported to have higher proliferative rates than diploid tumours. In this present study, however, there was no difference in the mean S-phase fraction of aneuploid as compared to diploid tumours and of responding as compared to non-responding tumours. The assessment of proliferation by flow cytometric measurement of Ki-S1 did, however, demonstrate a significant correlation between mean levels of expression and the degree of observed response. This correlation held for only the responding tumours which would suggest that overall response does not rely upon the rate of proliferation prior to treatment. However, in those tumours which do respond, greater degrees of response are associated with greater rates of cell proliferation. This does not correspond to the immunohistochemical findings from the pre-treatment biopsy material which failed to show predictive value for staining with Ki-S1. Indeed, there was no statistically significant correlation between flow cytometric and immunohistochemical measurement of Ki-S1 expression. The reason for this is unclear particularly since the same monoclonal antibody was used in both investigations. In fact, the results showed no significant correlations between the two methods of analysis for any of the individual markers studied. In the cases of measurement of c-*erb*B-2 and *P*-glycoprotein expression different antibodies, as necessitated by the different techniques used in the two methods, may partly explain the poor correlation. There

were also differences in tissue fixation with material for flow cytometry fixed in alcohol and formalin fixation used for immunohistochemistry. Perhaps more importantly, flow cytometric analyses are problematical in that varying amounts of stromal cells are included in the analyses whereas in immunohistochemical assessments only directly identifiable tumour cells are included. However, samples derived from fine needle aspiration do, theoretically, provide a better representation of the tumour as a whole, since the needle is passed through a relatively large volume of tumour during collection. Material for immunohistochemical studies, however, necessarily represents only a very small portion of tumour, allbeit in cross section.

On assessment by both techniques the expression of pS2 after tamoxifen treatment was shown to positively correlate with the degree of response. Interestingly, by flow cytometry the percentage of cells expressing pS2 prior to treatment correlated with subsequent degree of response but after treatment it was the mean cellular expression which was relatedto the prior response. In other words the proportion of tumour cells expressing pS2 predicts response but the degree of individual cellular expression after treatment reflects response. So that tumour cells are not recruited to express pS2 but those which do so are induced to express higher levels. pS2 expression is under oestrogenic control as, indeed, is the expression of c-*erb*B-2 according to some authors. Oestrogens have been shown to induce pS2 but inhibit c-*erb*B-2 expression in hormone sensitive cell lines. There was a positive correlation between response and the initial expression of c-*erb*B-2 and a significant decrease in expression of the marker in responding tumours. The results relating to the expression of pS2 and c-*erb*B-2 might suggest that the oestrogenic effects of tamoxifen are important in tumour response. It is not possible to determine whether these oestrogenic effects *per se* are important or whether they reflect the possession of fully functional oestrogen receptor mechanisms through which tamoxifen may exert other actions.

The numbers of both B-lymphocytes and Natural Killer cells present in pre-treatment FNA's, as measured by the numbers of cells expressing CD19 and CD56 antigens respectively, did show associations with response. Numbers of both cell types were significantly correlated with the degree of response to tamoxifen when the responding population only were assessed. The presence of both of these cell types within primary breast tumours have been shown in a previous study to be related to good overall prognosis (Lawry, 1993). This study did not, however, relate these parameters to the response to systemic treatment. Other studies have examined the effect of tamoxifen on the activity of Natural Killer cells isolated from venous blood samples. There appears to be an increase in activity after short-term treatment (one month) but a decrease in activity after longer periods of tamoxifen therapy (years) (Robinson, 1993).

Few conclusions could be drawn from the material obtained by FNA in tumours prior to undergoing primary treatment with chemotherapy as there were relatively few numbers and only a small variation in the degree of response recorded. Within these limitations, S-phase fraction and DNA ploidy prior to treatment did not predict for the clinical response seen. There were no significant associations with response noted for the other tumour antigens measured; c-*erb*B-2, *P* -glycoprotein and Bcl-2.

Fine needle aspirates taken during treatment allowed monitoring of changes in tumour cell cycle distribution and antigen expression. Overall S-phase fraction did not prove to be as reliable as Ki-S1 expression in demonstrating the expected decrease in cellular proliferation in responding tumours. The potential problems of S-phase measurements in treated tumours had been demonstrated in animal models and described above. BrdU uptake proved difficult to measure reliably on the relatively small amount of material obtained by FNA given that several other analyses were also required on each sample. Serial FNA's demonstrated a significant decrease in Ki-S1 expression in the midpoint (6 weeks) samples compared with those taken prior to therapy in tumours treated with tamoxifen. Similarly, a tendancy towards reduced expression was seen in the sample taken before the third cycle of treatment in patients given chemotherapy. However, although this may be a promising tool to monitor biological response at a relatively early stage in treatment, more tumours will need to be assessed before definite conclusions can be drawn. No clear trends in changes in cell cycle distribution were evident such that $G_{0/1}$ and G_2 blocks were not generally seen in tumours treated with tamoxifen and adriamycin respectively. Such effects of these drugs have previously been reported in cell culture systems and the failure to demonstrate these in this study may reflect the heterogenous nature of a primary breast carcinoma.

There were interesting changes in DNA content during treatment with two diploid tumours developing an aneuploid and a tetraploid histogram when treated with tamoxifen and one tumour demonstrating the development of an aneuploid peak when treated with chemotherapy. All three tumours demonstrated clinical response, but if these new peaks represent resistant clones of tumour cells it may well be that these patients develop recurrent disease during follow-up.

Clinical follow-up will be extremely important to determine the relationship with relapse-free and overall survival of the expression of tumour cellular antigens and cell cycle analyses. These relationships are probably as important as those relating to the short term response to primary therapy.

Adriamycin uptake was measured in the few tumours treated with chemotherapy that were studied. Two out of five tumours demonstrated a decrease in uptake with the

third cycle compared with the first and the remainder showed consistently low levels of fluorescence. This apparent reduction in adriamycin-associated intracellular flourescence may simply reflect poor quality specimens from treated tumours but could demonstrate the development of cellular resistance or more likely reflect poor vascularity and hence delayed access of the drug to the tumour. In an attempt to investigate this theory, doppler ultrasound scans performed on one of the patients studied demonstrated a complete loss of arterial signal after two cycles of treatment (data not shown). Future studies of adriamycin uptake may have to account for this probable delay in drug transport. It is uncertain whether the decrease in vascularity is a direct effect of adriamycin on the vasculature or on indirect effect of tumour cell death. The poor vascularity may prevent exposure of a proportion of the tumour cell population to the cytotoxic drug allowing continued proliferation and metastasis.

The rapid and often large response to chemotherapy in terms of reduction in tumour volume, in many cases after two cycles of treatment, made tumour sampling technically very difficult and produced poor quality specimens. This will limit future work with serial FNA samples in chemotherapy-treated tumours particularly when the primary tumour is relatively small prior to treatment.

Chapter 18: Conclusions

This thesis has attempted to examine the response of breast cancer to primary systemic therapy by a variety different methods and at several levels. It has confirmed the usefulness of ultrasound scanning to monitor macroscopic response in terms of reduction in tumour volume. Indeed it is the first study to report serial ultrasound measurements in patients treated with primary tamoxifen and has produced surprising results in that response can be detected after only a relatively short treatment period. The relevance of the speed of response of an individual tumour to primary tamoxifen with respect to overall patient survival will only be detemined after significant clinical follow-up. Ultrasound also gives an accurate assessment of the degree of response in terms of reduction in tumour volume. This may be particularly important in assessing various predictive markers since, increasingly, it is felt that the prime benefit of primary systemic therapy will be the avoidance of mastectomy after tumour shrinkage. This certainly appears to be borne out in the patients from the Trial of Primary Systemic Therapy who demonstrate little difference in survival between those treated conventially and those given primary drug treatment (although follow-up is short). To this end if the degree of likely response can be predicted before treatment then this would be the major selection criteria for primary drug therapy. In this thesis serial ultrasound scanning enabled the demonstration that whilst estimates of oestrogen receptor content both by ERICA and EIA correlated with overall response to primary tamoxifen only EIA estimations correlated closely with the degree of the observed response. None of the other markers assessed by immunohistochemistry predicted for the observed response either to hormone or chemotherapy. Flow cytometric analysis of a small sub-population of tumours, however, did suggest that the expression of c-*erb*B-2 predicts for both the overall and the degree of response to tamoxifen. The model, however, will allow further markers to be assessed and, indeed, archival material is available for future analysis from the majority of tumours whose clinical response has been documented.

An accurate measurement of tumour response also allows a better understanding of the response to systemic therapy at a cellular level. The changes in expression of a variety of cellular markers revealed some interesting trends. However, the relatively few patients receiving primary chemotherapy in the study population and the small variation in their observed responses has made it difficult to draw any very useful conclusions from this section of the work. In those tumours treated with tamoxifen, potentially the most interesting markers have been that of proliferation, Ki-S1, and that of resistance to cell death, Bcl-2. The examination of changes in the expression of

these two markers and the degree of response exhibited to tamoxifen have raised the possibility of two different pathways through which tamoxifen exerts its effects. Firstly through decreasing cellular proliferation, the classical cytostatic mechanism but also secondly through a distinct effect to reduce Bcl-2 expression. This may in turn lead to an increase in cell death by apoptosis. Further work is under way to confirm this finding through other methods of estimating rates of apoptosis and to understand the molecular pathways involved in translating exposure to tamoxifen into the protein changes seen by immunohistochemistry. The relationship between the changes in expression of *P* -glycoprotein and observed response, although surprising, may have significant clinical implications. If the expression of *P* -glycoprotein is induced in tumours that are resistant to tamoxifen then this drug may be contraindicated in patients who would be candidates for future treatment with chemotherapeutic agents which are susceptible to this resistance mechanism. This would also, therefore, highlight the need for more definitive predictive factors for tamoxifen response/resistance. The current trend for a combined approach to the systemic treatment of breast cancers with simultaneous (or closely separated) courses of tamoxifen and chemotherapeutic drugs may also be questioned. There are also large trials underway examining the use of tamoxifen as a preventative therapy in young women with several risk factors for the development of breast cancer (Powles, 1989). One wonders how chemosensitive such tumours will be if they have developed during, and are hence resistant to, the effects of tamoxifen. Because of the potential implications of this finding further work with the expression of *P* -glycoprotein and exposure to tamoxifen needs to be undertaken.

Much of this proposed future work could be performed profitably in animal model systems. The small studies reported here, the first work to our knowledge to describe serial fine needle aspirates from animal xenografts, confirm the potential of such systems. Ideally such work would be performed with xenografts taken from a variety of primary human breast tumours. However breast tumours remain notoriously difficult to xenograft and all attempts (unreported) made by the author to do so during the work for this thesis, despite a variety of different conditions, were unfruitful.

The potential promise of the combined techniques of fine needle aspiration and flow cytometry although giving useful results in the xenograft material only partly realised such potential in the human tumours studied. The limiting factors appeared to be of tissue quality and quantity, particularly at later time points in responding tumours. Surprisingly, there was not a single positive correlation between antigen expression as measured by immunohistochemistry and by flow cytometry, and although various theories have been discussed above this warants further investigation. Nevertheless, the significant positive correlations between the degree of response and pre-treatment

expression of both pS2 and c-*erb*B-2 are potentially important in the understanding of the mode of action of tamoxifen and should be examined further. Flow cytometric analyses allowed additional information relating to DNA content and cell cycle distribution to be obtained. One of the advantages of such techniques is the minimally invasive nature of fine needle aspiration. If the immunohistochemical changes noted in Ki-S1, Bcl-2, and *P* -glycoprotein expression are confirmed in other studies then further work on the expression of these antigens in fine needle aspirates early in treatment would be appropriate. Results reported here suggest that changes in Ki-S1 and *P* -glycoprotein could be noted by six weeks of treament with tamoxifen. Bcl-2 has not been examined by flow cytometry in fine needle aspirates in this study but the methodology does exist. It would be a significant advance if after six weeks of treatment (or perhaps earlier) one could predict, by changes in cellular antigen expression, that the desired volume reduction will not be achieved or that in apparently resistant tumours there was a risk of developing an element of chemoresistance.

This thesis, by the nature of the proposed content, has included a broad overview of methods of assessment of the response to primary therapy of breast tumours. It has, however, made significant observations of the macroscopic response to treatment, confirmed the power of standard predictors of response such as the oestrogen receptor and indicated new potential markers of response and resistance to primary therapy. It forms the basis for future, more detailed, work on individual parameters designed to optimise the use of the currently available, but unfortunately far from perfect, therapeutic options for the treatment of breast cancer.

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